Cloning and Characterization of a cDNA Involved in Porcine Exocrine Chloride Conductance

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Abstract

A heterogeneous population of apical membrane chloride channels is responsible for transepithelial secretion of chloride ions in the small intestine, thereby providing a driving force for fluid secretion into the intestinal lumen. Previous experimental characterization of one of these pathways led to the selection of a monoclonal antibody capable of significantly inhibiting conductive chloride uptake into ileal apical membrane vesicles.

This antibody was used to isolate a 0.94 kb cDNA clone, PG33, from a porcine intestinal expression library. A labeled probe constructed from this sequence identified a 2.7 kb band in a Northern blot of porcine parotid gland mRNA. Further clones were isolated through conventional oligonucleotide screening of the porcine library, as well as PCR-based screening of the porcine library and a human intestinal cDNA library. The total sequence cloned to date includes 2.3 kb, encoding a 679-amino acid translation product. The primary sequence bears significant homology to only two known genes: Ca-CC, a calcium-regulated chloride channel expressed in bovine tracheal epithelium, and Lu-ECAM-1, a lung endothelial cell adhesion molecule.

Expression of PG33 mRNA was detected by reverse transcriptase PCR in several porcine secretory epithelial tissues, including ileum, trachea, and the major salivary glands (parotid, submandibular, and sublingual). In situ hybridization studies confirmed the ileal and tracheal expression. In porcine ileal mucosa, PG33 mRNA was detected in both the crypt and villus epithelial regions, while in the trachea, expression was observed both in the surface epithelium and the submucosal glands.

Reverse transcriptase PCR studies demonstrated that the tissue expression of PG33 mRNA is distinct from that of the bovine chloride channel Ca-CC. Expression of Ca-CC appears restricted to bovine tracheal epithelium, and could not be detected in bovine ileum or parotid gland, nor in porcine trachea, ileum, or parotid gland. PG33 gene expression could not be detected in bovine tissues, though both PG33 and Ca-CC mRNA were detected in the human intestinal cell line T84 and the human airway cell line

HBE-1. Expression of the PG33 and Ca-CC genes in these two lines may depend upon the differentiation status of the cell.

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And to Derek, who, as always, strenuously resists categorization, and to whom I can only say: for all of the above. And more.

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List of Abbreviations

α-PC	α-phenylcinnamate
ABC	ATP-binding cassette
aGM_1	asialoganglioside 1
AMP-PNP	5'-adenosine (β,γ-imino) triphosphate
ATP	adenosine triphosphate
BAEC	bovine aortic endothelial cell
BAPTA	[1,2-bis(2)aminophenoxy]ethane N,N,N',N'-tetraacetic acid
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BSA	bovine serum albumin
Ca-CC	bovine tracheal calcium-regulated chloride channel
CaMK	calmodulin-sensitive protein kinases
cAMP	adenosine 3'-5'-cyclic monophosphate
CBAVD	congenital bilateral absence of the vas deferens
cDNA	complementary DNA
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
cfu	colony forming units
CHO	Chinese hamster ovary cells
CIAP	calf intestinal alkaline phosphatase
CPT-cAMP	8-(4-chlorophenylthio)adenosine cyclic 3'-5'-monophosphate
DAG	diacylglycerol
DEPC	diethylpyrocarbonate
DIDS	4,4'-diisothiocyanostilbene-2,2'-disulfonic acid
DME	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphate
DPC	diphenylamine-2-carboxylate
DTT	dithiothreitol
ECGS	endothelial cell growth supplement
EDTA	ethylenediamine tetraacetic acid - disodium salt
EGF	epidermal growth factor
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GC	guanylate cyclase
HEPES	N-2-hydroxylethylpiperazine-N'-2-ethanesulfonate
$Ins(1,4,5)P_3$	inositol 1,4,5-trisphosphate
$Ins(3,4,5,6)P_4$	D-*myo*-inositol 3,4,5,6-tetrakisphosphate
IPTG	isopropylthio-β-D-galactopyranoside
I-V	current-voltage relationship

kDa	kiloDaltons
Lu-ECAM-1	lung-endothelial cell adhesion molecule-1
mAb	monoclonal antibody
MDCK	Madin-Darby canine kidney cells
MMLV	Moloney murine leukemia virus
NBD	nucleotide binding domain
NBT	nitro-blue tetrazolium
NPPB	5-nitro-2-(3-phenylpropylamino)benzoic acid
ORCC	outwardly rectifying chloride channel
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PI	pancreatic insufficient
PITC	phenyl isothiocyanate
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PKG	cGMP-dependent protein kinase
pS	picoSiemens
PS	pancreatic sufficient
rENaC	rat epithelial sodium channel
RT PCR	reverse transcriptase PCR
SDS	sodium dodecyl sulfate
SITS	4-acetamido-4'-isothiocyanolstilbene-2,2'-suphonic acid
SRG	shark rectal gland
SSC	standard saline citrate
Sta	enteropathogenic bacterial heat-stable enterotoxin
TBS	Tris-buffered saline
TESPA	3-aminopropyltriethoxylsilane
thio-UTP	uridine 5' [α-thio] triphosphate
TMA	tetramethylammonium
TTBS	Tween-20 in Tris-buffered saline
UTP	uridine triphosphate
UTR	untranslated region
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

1.0 Introduction

The ability to control fluid movement from intracellular to extracellular compartments is determined by the type and location of ion channels in the plasma membrane. In secretory epithelia such as the proximal small intestine, apical membrane chloride channels allow the movement of chloride into the gut lumen in response to events which increase the intracellular chloride concentration above its equilibrium point.

While it has been clear for some time that several different intracellular signalling mechanisms control the exit of chloride ions, the identity of the channel or channels responsible for the majority of the chloride secretory response has been a matter of much debate. In the past decade, a number of chloride channels have been characterised in intestinal epithelia, including the cystic fibrosis transmembrane conductance regulator (CFTR), the outwardly rectifying chloride channel (ORCC), and ClC-2. It is not currently known, however, precisely how many chloride channels exist in the brush-border apical membrane, nor exactly how their activity is coordinated to ensure that the amount of chloride secretion is appropriate to the circumstances.

One particular conductive chloride pathway has been under investigation in this laboratory for some time. Studies involving a number of experimental approaches have tentatively identified a 90 kDa brush-border protein as a candidate protein for this chloride channel (Racette *et al.*, 1996). The kinetics of this pathway have been shown to be saturable (Forsyth & Gabriel, 1988), and the activity of the putative channel is sensitive to inhibition by α-phenylcinnamate (Forsyth & Gabriel, 1989b), though it is relatively insensitive to typical chloride channel blockers such as SITS or anthracene-9-carboxylate (Forsyth & Gabriel, 1988).

Since the purification of single proteins from membrane preparations is often technically extremely difficult, a homogenous preparation of this candidate protein had not been obtained. In order to ascertain its actual role in chloride conductance, a molecular biological approach was therefore undertaken.

The major aim of this project was to identify, through the use of an antibody inhibitory for small intestinal chloride conductance, the gene responsible for encoding the candidate protein. This part of the project was intended to provide a pure source of protein for tests of its functional abilities as a chloride channel. As well, the tissue expression of this gene was to be investigated, both to compare its expression with that of known chloride channels, and to correlate the expression of the specific message with the location of the antigen against which the anti-chloride conductive antibody had been raised.

2.0 Literature Review

2.1 Morphology of the Small Intestine

The mammalian small intestine functions as both a secretory and as an absorptive organ in order to meet the demands placed upon it by widely varying daily fluid loads. Normally, the absorptive aspect predominates: though nearly ten litres of fluid pass through the average human small intestine each day, only approximately one litre enters the large intestine from the ileum. The secretory ability of the small intestine, while less dramatic, is also critical, as it contributes to the osmotic environment required for intestinal digestion and absorption of nutrients. The importance of fluid secretion in the gut is illustrated by the pathological consequences of its excess, which occurs in secretory diarrhea, or its insufficiency, which occurs in cystic fibrosis.

The secretory and absorptive capacities of the small intestine appear to be divided both histologically and anatomically. Both are a function of the mucosal layer of the intestinal wall. The mucosa consists of the surface epithelium, which is exposed to the contents of the lumen, along with an underlying layer of connective tissue called the lamina propria, and a thin layer of smooth muscle, the muscularis mucosae. The surface epithelial layer is functionally divided into crypts, which originate deep in the intestinal wall, and villi, which are fingerlike projections extending outwards into the lumen. Secretion is held to be a function of newly-produced epithelial cells in the crypts (Welsh *et al.*, 1982); these cells differentiate into absorptive cells as they migrate upwards before they are eventually sloughed off from the villus tip into the intestinal lumen. As well, while the proximal regions of the small intestine such as the duodenum are predominantly secretory, more distal regions such as the ileum appear to act in a predominantly absorptive fashion.

The key to these secretory and absorptive abilities lies in the polarized organization of the epithelial cells lining the intestinal wall. In a polarized cell, the protein and even the lipid compositions of the apical membrane are different from that of the basolateral membrane (Handler, 1989). As a result, some functions of the cell, especially transport functions, are segregated to the parts of the plasma membrane containing the appropriate proteins. In the case of ion transport, the proteins responsible for the movement of electrolytes are differentially targeted to the apical and basolateral membranes, and then restricted to their respective membrane domains by interactions with cytoskeletal elements and by the barrier formed by intercellular tight junctions (Handler, 1989; Rodriguez-Boulan & Salas, 1989). This inequal distribution of transport molecules between the apical and basolateral membranes is responsible for the vectorial movement of electrolytes across epithelial cells. Depending on the membrane distribution of transport proteins, movement may be either serosal-to-mucosal or mucosal-to-serosal. In the first instance, the cell is acting in a secretory manner; in the second, it is absorptive.

The electrical driving force for this vectorial movement in polarized cells is established by the actions of the basolateral Na^+/K^+ ATPase, which couples the inward transport of K^+ and the outward transport of Na^+ to the hydrolysis of ATP (see Figure 2.1). This results in an inwardly-directed Na^+ concentration gradient, which the basolaterally-located Na-K-2Cl cotransporter uses to move Na^+, K^+, and Cl^- into the cell from the extracellular fluid in an electrically neutral fashion. K^+ leaves the cell passively through basolateral K^+ channels while Cl^- exits through apical membrane channels along its electrochemical gradient. The lumen-negative transepithelial potential thus generated provides an electromotive force for serosal-to-mucosal Na+ movement through a paracellular pathway.

2.2 Basolateral Membrane Transporters In Secretory Epithelia

2.2.1 Na/K ATPase

The Na/K ATPase, also called the Na/K pump, is a ubiquitous protein that helps preserve transmembrane ionic gradients by simultaneously regulating both the

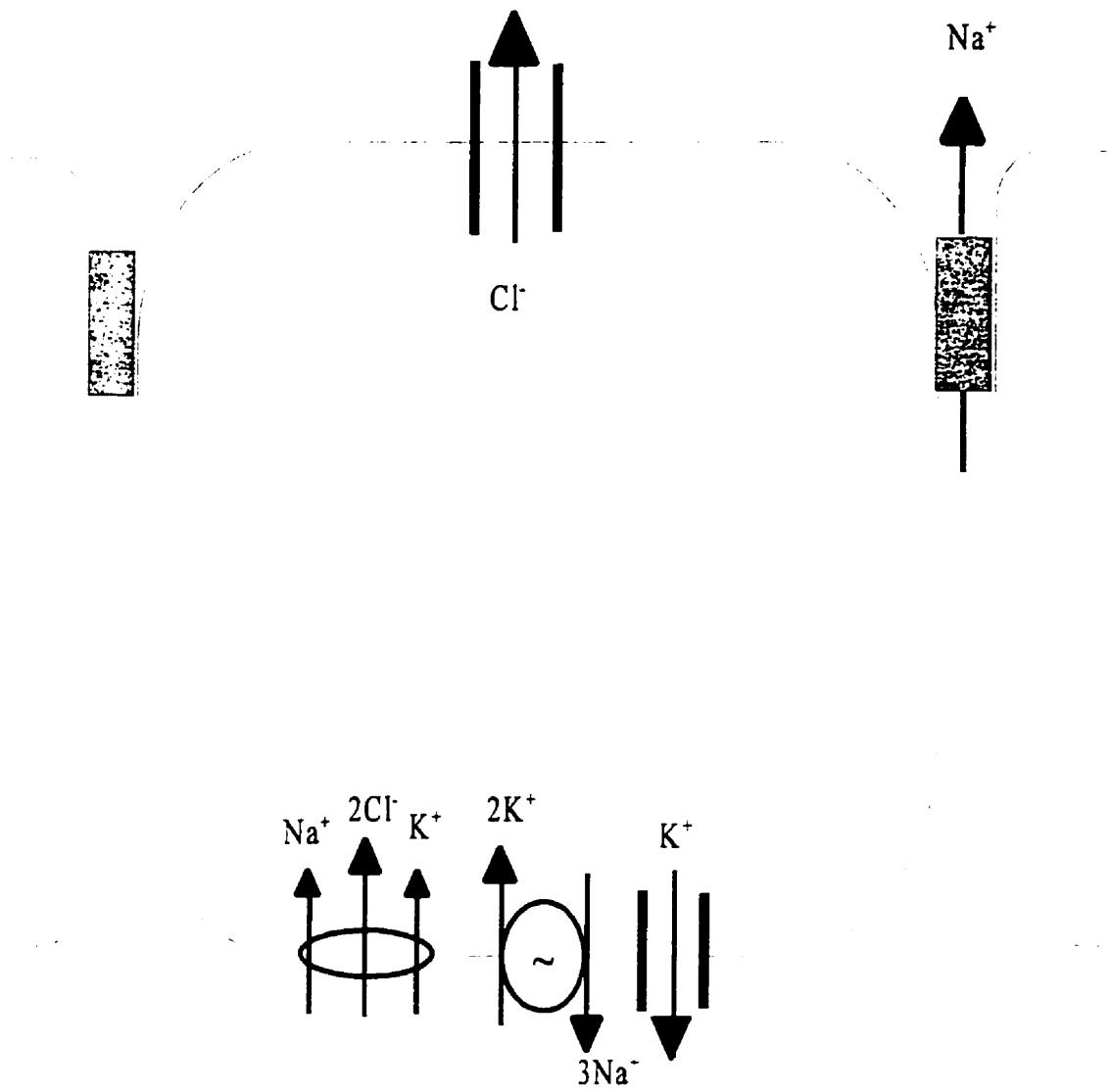


Figure 2.1 Selected ion transport mechanisms in a secretory cell. Tight junctions are indicated by shaded boxes. The activity of the Na^+/K^+ pump requires ATP hydrolysis (~).

intracellular and extracellular levels of sodium and potassium. The transport of these ions against their concentration gradients is carried out through the expenditure of chemical energy in the form of ATP hydrolysis. In this manner, for each molecule of ATP split, 3 Na^+ can be transported out of the cell, and 2 K^+ can be transported into the cell. This results in the creation of a high intracellular and a low extracellular K^+ concentration, and a high extracellular and low intracellular Na^+ concentration. The movement of more positively charged ions out of the cell than into the cell with each ATP hydrolyzed results in the accumulation of a net negative charge on the interior of the cell membrane. This charge imbalance helps establish what is known as the membrane potential, which is essentially the potential energy due to the separation of positive and negative charges on either side of the plasma membrane. In epithelial cells, the membrane potential is usually between -35 and -40 mV.

The electrochemical gradient generated by the Na/K pump allows a number of energetically unfavourable transport processes to be linked to the movement of sodium ions without requiring direct ATP hydrolysis by the protein transporters. This process is known as secondary active transport, and includes the sodium-linked cotransport of nutrients such as glucose or amino acids as well as cotransport of other electrolyte species such as K^+, Cl^-, and H^+. Preservation of the electrochemical gradient may require anywhere from 5 to 40% of the available energy of a cell, depending on the metabolic activity level of that cell (Ismail-Beigi & Edelman, 1971; Ewart & Klip, 1995).

The Na/K pump is an integral membrane protein consisting of two joined subunits. The 112 kDa α subunit is the catalytic subunit and contains a number of membrane-spanning domains (Skou, 1992; Ewart & Klip, 1995). Contained within the α subunit are the binding sites for Na^+, K^+, Mg^{2+} ATP, and the inhibitory cardiac glycoside ouabain (Geering, 1991; Ewart & Klip, 1995). K^+ and ouabain bind on the extracellular side of the α subunit, while Na^+ and MgATP bind on the cytoplasmic surface of the subunit. The smaller, associated β subunit has a single transmembrane domain with a glycosylated extracellular portion (Geering, 1991; Fambrough *et al.*,

1994)). The molecular mass of the β subunit varies depending on the amount of glycosylation present, but the mass of the core protein is approximately 35 kDa (Skou, 1992; Ewart & Klip, 1995). The precise function of the β subunit is not currently known, though assembly of the two subunits appears to be required for protein maturation, transport to the plasma membrane, and subsequent enzyme activity (Geering, 1991). Each of the subunits occurs in a number of isoforms, the distribution of which is tissue-specific; the most commonly occurring form of the enzyme, however, is an $\alpha_1\beta_1$ association (Sweadner, 1989; Ewart & Klip, 1995)

Regulation of Na/K pump activity can be divided into acute and long-term phases. Acute or short-term regulation generally refers to transient changes in the activity of existing pump units, while long-term regulation usually involves an increase in the synthetic rate of the Na/K pump due to a stimulation of transcriptional and translational events, as well as changes in the stability of pump subunit transcripts (Jorgensen, 1986; Ewart & Klip, 1995).

Acute regulation of Na/K ATPase activity has traditionally been considered to reflect the intracellular levels of Na^+ (Jorgensen, 1986), which is the rate-limiting species for the pump. More recently, receptor-mediated increases in a number of intracellular second messengers such as cAMP, diacylglycerol (DAG), and arachidonic acid have also been implicated in short-term pump regulation (Bertorello & Katz, 1993; Bertorello & Katz, 1995; Ewart & Klip, 1995). The actions of these messengers appear to vary depending on the tissue studied (Bertorello & Katz, 1993; Bertorello & Katz, 1995; Ewart & Klip, 1995); consequently, the final effect on pump activity may be stimulatory or inhibitory. Finally, cytoskeletal elements have also been shown to influence Na/K pump activity, in that G, or monomeric, actin can stimulate pump activity, while F, or filamentous, actin cannot (Bertorello & Cantiello, 1992). Possible interactions between actin, cellular protein kinases such as PKA and PKC, and the Na/K pump are still unclear (Bertorello & Katz, 1995).

2.2.2 Na-K-2Cl Cotransporter

Sodium-linked cotransport of chloride ions provides a mechanism for increasing

the intracellular level of chloride above its electrochemical equilibrium without requiring additional energy beyond that expended by the Na/K ATPase. The electrochemical gradient in turn determines the direction of chloride movement from the cell; in the case of secretory epithelia, chloride ions exit through apically-located chloride channels.

Electroneutral cotransporters include the Na-Cl cotransporter, the Na-K-2Cl cotransporter, and the K-Cl cotransporter (Haas, 1994). Of these, the Na-K-2Cl cotransporter is the best characterised cotransporter in secretory epithelia. This cotransporter binds Na^+, K^+, and Cl^- in an interdependent fashion, in that all three ions must be present and bound to the cotransporter before the transport process can occur (Lytle & McManus, 1986). The stoichiometry for nearly all such cotransporters is $1Na^+$: $1K^+$: $2Cl^-$ (O'Grady *et al.*, 1986; O'Grady *et al.*, 1987). Another characteristic of the Na-K-2Cl cotransporters is their sensitivity to loop diuretics, or sulfamoylbenzoic acid derivatives such as furosemide and bumetanide (O'Grady *et al.*, 1987; Haas 1994). Loop diuretics have been hypothesized to inhibit cotransport by preventing the interaction of the final chloride with its binding site on the protein, thereby blocking the ability of the cotransporter to undergo the necessary conformational change for ion transport (Hedge & Palfrey, 1992).

The driving force for ion cotransport is established by the sum of the chemical gradients of all the electrolyte species involved (Haas, 1994). In other words, the direction and amount of cotransport is dictated by the chemical gradients for Na^+, K^+, and Cl^-. The sodium and chloride gradients are inwardly directed, while the potassium gradient is outwardly directed; as a result, because two chloride ions are transported for each sodium and potassium, the effects of the chloride gradient predominate in determining the direction of ion transport.

A variety of Na-K-2Cl cotransporter isoforms have been characterised in epithelial tissues, and can be categorized as either members of apical absorptive or basolateral secretory families (Payne & Forbush, 1995). Members of the basolateral secretory family are all homologous to a cotransporter originally identified in shark

rectal gland (SRG), NKCC-1 (Xu *et al.*, 1994; Payne & Forbush, 1995); this isoform has a wide tissue distribution, including intestinal epithelium (Xu *et al.*, 1994). The apical absorptive isoform, on the other hand, appears to be restricted to epithelial cells of the kidney (Payne & Forbush, 1994).

The protein structure for NKCC-1 has been deduced from the SRG cDNA encoding this protein (Xu *et al.*, 1994). The mass of the core protein is roughly 130 kDa, while the mass of the fully glycosylated mature protein varies depending on the tissue and species. Based on amino acid sequence, the NKCC proteins are all predicted to contain large cytosolic N- and C-terminal domains flanking 12 putative membrane-spanning segments (Xu *et al.*, 1994; Haas, 1994; Payne & Forbush, 1995). Several consensus sequences for N-linked glycosylation have been identified on one of the extracellular loops between transmembrane segments (Xu *et al.*, 1994).

Activity of the Na-K-2Cl cotransporter is subject to a number of regulatory influences. These influences may either directly affect cotransporter activity, or they may be secondary to some event that activates apical chloride channels and therefore requires an increased basolateral chloride influx (Haas *et al.*, 1993; Haas, 1994). In many cases, it is difficult to distinguish the two.

Elevation of intracellular cAMP, and therefore activation of PKA, has been associated with increased cotransporter activity (Dharmsathaphorn *et al.*, 1985; Lytle & Forbush, 1992; Haas *et al.*, 1993; Haas, 1994; Payne & Forbush, 1995). It is not currently clear whether the activation due to PKA is due to a direct kinase action upon the cotransporter, or whether PKA activates some other regulatory component that in turn activates the cotransporter (Haas, 1994; Payne & Forbush, 1995).

Cytoskeletal elements have also been implicated in the regulation of the Na-K-2Cl cotransporter (Haas, 1994; Payne & Forbush, 1995). Reagents that maintain actin in monomeric or short filamentous form appear to activate the cotransporter, while compounds that block actin disassembly from the longer F-actin form inhibit cotransporter activity (Matthews *et al.*, 1994; Matthews *et al.*, 1997) .

2.2.3 K Channels

The basolateral K^+ conductance performs a number of functions in secretory epithelia. This pathway permits exit of the K^+ brought into the cell by the Na/K ATPase and the Na-K-2Cl cotransporter. Upregulation of K^+ conductance hyperpolarizes the cell, increasing the stimulus for Cl^- exit via apical chloride channels. Many secretagogues that increase free cytosolic Ca^{2+} levels, for example, stimulate net chloride secretion by stimulating the K^+ conductive pathway. The potassium channel blocker barium (Ba) prevents chloride secretion, on the other hand, by inhibiting K^+ exit from the cell through K^+ channels (Welsh, 1983; Smith & Frizzell, 1984).

Coordination of potassium movement with chloride exit from epithelial cells is essential during salt secretion in order to maintain the balance of charge and mass within the cell, thereby keeping cell volume constant (Dawson & Richards, 1990). For each cycle of the Na/K ATPase, six chloride ions can be secreted at the apical membrane (see Figure 2.1 for stoichiometry). In this simplified model, the sodium ions entering via the cotransporter are recycled by the Na/K pump. For each ATP hydrolyzed, only two K^+ are brought in while 3 Na^+ are transported out of the cell; as a result, if all of the chloride ions entering the cell are secreted at the apical membrane, charge balance can only be preserved if the remaining five potassium ions exit via basolateral channels.

If chloride secretion and potassium conduction are linked, then, stimuli which cause an increase in net chloride secretion should also activate potassium channels. These K^+ channels may be classified as either channels which respond to increases in cytosolic free Ca^{2+} or those which activate in response to increases in cyclic nucleotides such as cAMP (Dawson & Richards, 1990; Barrett, 1993; Breitwieser, 1996), cAMP and Ca^{2+} being two of the major intracellular messengers for chloride secretagogues. In fact, calcium-linked chloride secretion has traditionally been held to be driven primarily by the activation of basolateral calcium-regulated potassium channels (Barrett, 1993).

The identity of these K^+ channels has been complicated by the technical difficulty of obtaining electrophysiological records of channels in the basolateral

membrane (Dawson *et al.*, 1990). A number of epithelial K^+ channels have been characterised, however, based on measurable biophysical properties such as single-channel conductance, sensitivity to common potassium channel blockers, and current-voltage (I-V) relationship.

A class of large-conductance K^+ channels have been identified in many secretory epithelial cells, including rat small intestine (Morris *et al.*, 1986) and rabbit distal colon (Loo & Kaunitz, 1989). These channels tend to have very high single-channel conductances (between 100-300 pS in symmetrical KCl solutions). Consequently, the usual descriptive term for this class of channels is "maxi" K^+ channels. These channels are also usually calcium-activated and sensitive to blockade by barium ions (Latorre & Miller, 1983; Morris *et al.*, 1986; Loo & Kaunitz, 1989; Dawson & Richards, 1990).

Several groups of investigators (Devor & Frizzell, 1993; Tabcharani *et al.*, 1994a; Tabcharani *et al.*, 1994b; Roch *et al.*, 1995) have reported the presence of smaller-conductance, inwardly-rectifying K^+ channels in the basolateral membrane of T84 intestinal epithelial cells similar to a channel previously characterised in dog tracheal epithelial cell cultures (Welsh, 1985). In addition to the lower single-channel conductances, these channels are all calcium-activated and insensitive to barium blockade, though varying degrees of inhibition of this channel by charybdotoxin were reported.

There is some debate as to whether there is overlap in the regulation of K^+ channels by cAMP and calcium, or in other words, whether both cAMP and free calcium levels have the ability to control the same channel. In rabbit distal colon, the calcium-activated maxi-channels also responded to cAMP (Loo & Kaunitz, 1989), albeit with very different activation kinetics. Additionally, cAMP-associated activation of the smaller-conductance, inwardly-rectifying K^+ channels has been reported by two groups (Baro *et al.*, 1994; Tabcharani *et al.*, 1994b). In both cases, however, cAMP-sensitivity of the K^+ channel was demonstrated only in the presence of high levels of free calcium (Baro *et al.*, 1994; Tabcharani *et al.*, 1994b), which led to the suggestion that this cAMP-associated activation is not completely independent of a calcium-

regulated mechanism (Devor *et al.*, 1996a; Devor *et al.*, 1996b). Hence, these studies cannot be said to disprove earlier proposals that cAMP and Ca^{2+} regulate separate basolateral K conductances (McRoberts *et al.*, 1985; Devor *et al.*, 1996a; Devor *et al.*, 1996b).

2.3 Apical Membrane Chloride Channels In Secretory Epithelia

2.3.1 Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessive disorder affecting nearly 1 in 2000 live births, making it one of the most common genetic disorders recognised (Welsh *et al.*, 1995). The cystic fibrosis allele occurs in the Caucasian population at a frequency of 1 in 25; its frequency in black and Asian populations, and therefore the CF disease phenotype, is much lower (Welsh *et al.*, 1995).

CF has been traditionally identified by the production of excessively salty sweat in affected individuals. More seriously, CF is characterised by a high incidence of neonatal morbidity due to intestinal obstructions, and an extremely high childhood and adolescent mortality rate predominantly due to pulmonary infections. Although historically survival of CF individuals beyond childhood has been rare, recent therapeutic advances have resulted in an increase in the mean life expectancy to 29 years (Welsh *et al.*, 1995; Wood, 1997).

Cystic fibrosis affects a number of epithelial tissues, many of which perform exocrine functions. As indicated above, increased sweat electrolyte concentration is a clinical diagnostic factor for CF, since the sweat glands are affected in nearly all CF patients (Wood, 1997). The respiratory system is also affected in the majority of CF patients, though the clinical severity of pulmonary symptoms may vary from mild to extremely severe. Common signs of CF lung disease include the production of abnormally thick mucus in the respiratory tract, which predisposes the individual to chronic and recurrent pulmonary infections, in particular with organisms such as *Pseudomonas aeruginosa* and *Burkholderia cepacia* (Wood, 1997). In CF patients, pulmonary function may deteriorate progressively over time as a result of recurrent

infections with these and other opportunistic organisms, often leading to end-stage lung disease.

The digestive system is commonly affected, though again, the severity of the clinical manifestations in this system varies from patient to patient. CF patients can be classified as either pancreatic sufficient (PS) or pancreatic insufficient (PI) based on the integrity of the exocrine pancreatic ductal structure and hence the ability to secrete adequate levels of digestive enzymes into the duodenum as required (Marino & Gorelick, 1992). Pancreatic insufficient patients require enzyme supplements to properly digest nutrients, especially dietary lipids. The gastrointestinal tract itself may be affected: 10 to 15% of CF newborns experience a condition known as meconium ileus (Marino & Gorelick, 1992). In these infants, abnormally thick mucus blocks the GI tract, and removal of the obstruction usually requires medical intervention (Welsh *et al.*, 1995).

Other organs commonly affected by cystic fibrosis include those of the reproductive system. Until recently, CF individuals rarely survived to reproductive maturity, and as a result infertility was not immediately seen to be an overt consequence of CF. With increasing survival times, however, it became apparent that over 95% of CF males have decreased fertility rates, and that this infertility is related to a syndrome known as congenital bilateral absence of the vas deferens (CBAVD) (Chillon *et al.*, 1995; Welsh *et al.*, 1995; Tsui & Durie, 1997). Reproductive structures are also affected in CF females, resulting in low fertility rates compared to non-CF females (Welsh *et al.*, 1995).

2.3.1.1 Cellular Basis of CF Defect

Instances of cystic fibrosis-like disorders have been reported for several centuries. CF was only described as a distinct clinical syndrome, however, in 1938 (Anderson, 1938), though not until 1953 was the connection made between the presence of high-salt sweat and the disease cystic fibrosis (diSant'Agnese *et al.*, 1953). Also during this time interval, pedigree analysis demonstrated the autosomal recessive nature

of CF, indicating that the disease is inherited in a single-gene or classically Mendelian fashion (Anderson & Hodges, 1946).

Not until the early 1980s was the cellular defect underlying the disease pathology finally identified. The reabsorptive ducts of sweat glands (Quinton, 1983) and airway epithelial cells (Knowles & Boucher, 1981) from CF patients were found to have greater transepithelial potential differences than those from normal subjects; the defect was furthermore identified as an impermeability of the epithelials cells to chloride ions (Quinton, 1983). More specifically, affected tissues showed an inability to respond to β-adrenergic stimuli (Knowles & Boucher, 1983; Sato & Sato, 1984; Welsh & Lietdke, 1986; Frizzell *et al.*, 1986), though responses to cholinergic muscarinic agonists, which act by increasing intracellular calcium levels, were found to be normal in most CF tissues (Sato & Sato, 1984; Frizzell *et al.*, 1986; Widdicombe, 1986; Boucher *et al.*, 1989). Agonist-induced elevation of the intracellular second messenger cAMP was normal in CF tissues (Sato & Sato, 1984; Boucher *et al.*, 1986). This implied that the defect might lie in the response of an ion channel to cAMP-dependent stimuli, rather than in the production system of this second messenger.

The identity of this chloride channel was obviously of great interest. An outwardly rectifying chloride channel (ORCC) had been identified in tracheal epithelium (Welsh, 1986) that could be stimulated by isoprenaline, a cAMP-elevating agent (Welsh & Lietdke, 1986). This channel was also shown to open in response to strong depolarizing voltages (Hwang *et al.*, 1989). Activation in response to purified catalytic PKA plus ATP could consistently be demonstrated in membrane patches from normal airway epithelial cells, but not in patches from CF airway epithelia (Schoumacher *et al.*, 1987; Li *et al.*, 1988), though exposure of the CF patches to depolarizing voltages confirmed the presence of functional channels within the patches. Channel kinetics were identical in normal and in cystic fibrosis airway cells. A defect in this outwardly rectifying channel, therefore, was proposed to be responsible for the lack of chloride conductance in cystic fibrosis cells (Guggino, 1993).

2.3.1.2 Cloning of the CF Gene

As an autosomal recessively-inherited disease, cystic fibrosis is likely to result from a defect in a single gene, and hence a single protein product of such a gene. Because the defective protein in CF was thought to be a chloride channel, and since membrane proteins are notoriously difficult to purify to homogeneity, identification of the CF gene was undertaken in order to bypass these technical difficulties, and to allow the examination of the CF protein.

The CF gene had been mapped, through large-scale linkage analysis studies, to a single locus on the long arm of chromosome 7 (Knowlton *et al.*, 1985; Wainwright *et al.*, 1985; White *et al.*, 1985). Extensive restriction mapping and cloning studies using a variety of techniques further narrowed the location of the CF locus to a 250-kb region between the chromosomal markers *MET* and *D7S8* (Rommens *et al.*, 1989; Riordan *et al.*, 1989). Several overlapping cDNA clones corresponding to this gene were isolated from a number of epithelial cell libraries (Riordan *et al.*, 1989), and one of these was used as a probe to ascertain the size of the mRNA transcript in various tissues affected by CF. The size of the CF transcript was 6.5 kb in all of the tissues examined (Riordan *et al.*, 1989).

Based on the nucleotide sequence of the cDNA, the open reading frame (ORF) of the CF transcript appeared to code for a protein containing 1480 amino acids. Hydropathy analysis of this primary amino acid sequence predicted that the CF protein contained two repeated motifs, each containing six hydrophobic regions that might span the cell membrane. In addition, each of these motifs also contained a region in which consensus sequences for nucleotide (ATP) binding were found. Intriguingly, the predicted amino acid sequence of the CF protein was highly homologous, not to previously identified ion channels, but to a family of proteins known as the ATP-binding cassette (ABC) transporter superfamily (Riordan *et al.*, 1989). This family contains proteins such as the multidrug resistance-associated P-glycoprotein and the yeast STE export system, and is functionally characterised by the ability of its members to use ATP hydrolysis to drive substrate transport across the cell membrane (Riordan *et*

al., 1989).

The amino acid sequence for the CF protein product differs in one significant way from other members of the ABC family, containing an additional region not found in any other ABC transporter. This region contains many positively and negatively charged amino acids, as well as a number of consensus sites for protein phosphorylation by kinases such as PKA and PKC (Riordan *et al.*, 1989). Because of these potential phosphoacceptor sites, this region was named the R (for regulatory) region by the original investigators (Riordan *et al.*, 1989).

Because the CF gene appeared to encode a protein more closely related to transporter-type proteins than to any classical ion channel, the CF protein product was named the cystic fibrosis transmembrane conductance regulator, abbreviated CFTR (Riordan *et al.*, 1989), until its function could be more clearly defined.

2.3.1.3 The CF Gene Defect

Genetic analysis of the specific mutations in a large number of CF patients revealed that, in the majority of cases, the CF gene defect involves the deletion of a codon corresponding to a phenylalanine residue at position 508 (ΔF508) in the CF protein product (Kerem *et al.*, 1989). Approximately 70% of the CF chromosomes investigated to date bear this mutation (Kerem *et al.*, 1989; Welsh *et al.*, 1995). Haplotype analysis suggests that the widespread existence of this mutation is due to an original single mutational event which occurred between 50 000 and 60 000 years ago (Morral *et al.*, 1994). Several hundred other mutations of the CF gene have been identified, all of which exist at much lower frequencies than the ΔF508 defect (Welsh *et al.*, 1995). These other mutations include a wide variety of gene defects, including missense, frameshift, and termination mutations (Welsh *et al.*, 1995). Many CF patients are compound heterozygotes, bearing a different mutation on each affected chromosome (Kerem *et al.*, 1989; Welsh *et al.*, 1995).

The functional consequences of the ΔF508 mutation have been studied to determine the nature of the CFTR defect in cAMP responsiveness. ΔF508 CFTR, while

synthesized normally in the endoplasmic reticulum, does not appear in CF cells in the mature, fully glycosylated form (Cheng *et al.*, 1990). This failure to mature normally has been attributed to a defect in protein processing within the Golgi complex, and as a result, little or no ΔF508 CFTR appears in the cell membrane (Cheng *et al.*, 1990). However, this processing defect has been reported to be temperature-dependent. ΔF508 CFTR expressed in nonepithelial cells incubated below 30°C, a lower temperature than normal, not only appears in the apical membrane, but also exhibits channel activity (Denning et al., 1992).

The hundreds of documented CF mutations can be classified into four or five categories, based on the effect of a given mutation on protein function. The first category, class I, describes mutations which prevent normal biosynthesis of CFTR (Welsh & Smith, 1993; Zielenski & Tsui, 1995). Class II mutations include the ΔF508 mutation, and contains all those mutants which are improperly processed (Welsh & Smith, 1993; Zielenski & Tsui, 1995). Cells with class I or II mutations have little or no CFTR in the cell membrane. Because chloride secretion is virtually absent from these cells, class I and class II mutations are generally associated with severe clinical syndromes (Zielenski & Tsui, 1995).

While proteins with class III mutations can be detected in the plasma membrane, these proteins are unresponsive to regulation by intracellular ATP (Welsh & Smith, 1993; Zielenski & Tsui, 1995). Hence, these CFTR mutants are incapable of functioning as chloride channels despite their physical presence in the plasma membrane.

Class IV mutants reach the cell membrane and respond normally to PKA and ATP stimulation, but with diminished channel conductance or altered channel gating (Welsh & Smith, 1993; Zielenski & Tsui, 1995). Finally, class V describes those mutations which result in the formation of normal CFTR, but at much lower levels than in non-CF cells (Zielenski & Tsui, 1995).

2.3.1.4 Functions of CFTR

2.3.1.4.1 CFTR as a Chloride Channel

Because the predicted protein structure of CFTR appeared more closely related to solute pump proteins than to electrodiffusive ion channels, there was considerable debate as to whether CFTR could act as a chloride channel. A number of studies were undertaken to resolve this controversy, and within a few years of the cloning of the CF gene, a considerable amount of evidence had been amassed to support the hypothesis that CFTR is, in fact, a chloride channel.

Complementation of CF airway epithelial cells with a functional copy of the CF gene, for example, corrected the defect in the cAMP-regulated chloride conductive pathway (Rich *et al.*, 1990) in these cells. Likewise, expression of the wild-type CFTR in a cystic fibrosis pancreatic cell line (CFPAC-1) also corrected the chloride transport defect (Drumm *et al.*, 1990). While these studies did not prove that CFTR is a chloride channel, they confirmed the functional association of the CF gene product with the cystic fibrosis conductance defect.

Other studies were able to localise CFTR to the apical membrane of epithelial cells (Gregory *et al.*, 1990), which supports the association of CFTR with an apical chloride conductive pathway. Furthermore, expression of CFTR in a number of non-epithelial cell lines such as Chinese hamster ovary (CHO) cells, NIH 3T3 fibroblasts, and Sf9 insect cells, among other, resulted in the appearance of cAMP-regulated chloride currents (Anderson *et al.*, 1991a; Kartner *et al.*, 1991). The biophysical properties of these currents in non-epithelial cells were very similar to the properties of a chloride current previously characterised in whole-cell recordings of intestinal and pancreatic epithelia (Gray *et al.*, 1989; Cliff and Frizzell, 1990; Tabcharani *et al.*, 1990; Bear & Reyes, 1992).

Surprisingly, these properties were very different than those which had been reported in previous investigations; while the protein that was thought to be defective in CF was outwardly rectifying, with a single-channel conductance between 30 and 50 pS at 0 mV, the channel that appeared in these complemented cells had a much smaller

single-channel conductance (5-10 pS) and exhibited a linear current-voltage relationship (Anderson *et al.*, 1991a; Kartner *et al.*, 1991).

Other properties characteristic of the channel appearing in CFTR expression systems included a halide permeability sequence of $Br^- > Cl^- > I^-$ (Bell & Quinton, 1992; Cliff and Frizzell, 1994) and an insensitivity to the chloride channel blocker 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS). This channel was, however, sensitive to the chloride channel blockers diphenylamine-2-carboxylate (DPC) and glibenclamide, and this pharmacological profile can be used to distinguish CFTR from other apical membrane chloride channels (Cliff & Frizzell, 1994).

One of the studies cited as providing definitive support for the role of CFTR as a chloride channel involved the purification of recombinant CFTR from insect Sf9 cells in which CFTR had been overexpressed. The purified protein was then reconstituted into planar lipid bilayers, where it exhibited an anion-selective single-channel activity that could be stimulated by the addition of ATP along with the catalytic subunit of PKA (Bear *et al.*, 1992). Hence, CFTR in the absence of any other cell-associated factors can indeed act as a regulated chloride channel. Furthermore, the biophysical characteristics of the channel studied in all of these reports were very similar, and consistent with a channel which is relatively quiescent in unstimulated cells, but which becomes activated upon exposure to cAMP agonists. This behaviour correlates with the properties of the chloride current originally reported to be defective in CF, and most investigators now accept that this small linear chloride channel corresponds to the CF gene product.

2.3.1.4.2 CFTR as a Regulator of Other Proteins

2.3.1.4.2.1 Regulation of ORCC

If CFTR is a small-conductance linear chloride channel, but the channel originally implicated in CF has a larger conductance and is outwardly rectifying, what then is the relationship between CFTR and this ORCC?

Several suggestions were initially made about the nature of this relationship, including the hypothesis that CFTR and the ORCC may be splice variants of the same

protein. Evidence contradicting this hypothesis includes the report that expression of CFTR message and the functional presence of the ORCC do not correlate in several cell lines, including the intestinal T84 and CaCo-2 lines, the pancreatic line PANC-1, and the airway epithelial cell line 9HTEo- (Ward *et al.*, 1991). In addition, transfection of CF bronchial epithelial cells with the wild-type CFTR gene not only results in the appearance of the characteristic CFTR currents (small-conductance, with a linear current-voltage relationship), but also corrects the defect in cAMP sensitivity of the larger, outwardly-rectifying chloride channel (Egan *et al.*, 1992).

Finally, separate identities for the ORCC and CFTR were definitively established when an animal model for CF became available. CF 'knockout' mice were created by replacing the wild-type CFTR gene in embryonic stem cells with a truncated version of the CFTR gene; these stem cells were then used to breed a population of mice homozygous for this defective CFTR gene (*cftr* -/- mice) (Koller *et al.*, 1991; Snouwaert *et al.*, 1992). The presence of functional ORCC in CF knockout mice could be demonstrated by subjecting membrane patches excised from nasal epithelial cells to strong depolarizing voltages. These same outwardly-rectifying channels could not be activated by exposure to PKA and ATP, although the ORCC was responsive to PKA and ATP in patches from normal cells (Gabriel *et al.*, 1993). Hence, in the absence of functional CFTR, the ORCC lacks sensitivity to protein kinase A despite the physical presence of the channel in the membrane of CF cells.

2.3.1.4.2.2 CFTR Regulation of ENaC

In cystic fibrosis airway epithelial cells, although chloride conductance is lower than normal, the basal rate of sodium absorption across the apical membrane is greater than in normal cells (Knowles *et al.*, 1981; Knowles & Boucher, 1983; Boucher *et al.*, 1986; Duszyk & French, 1989; Grubb *et al.*, 1994). This hyperabsorption appears to be caused by an increased sodium permeability secondary to an increase in the open probability of apical sodium channels (Chinet *et al.*, 1994). Unusually for airway epithelia, β-adrenergic stimuli further increase sodium absorption in CF but not in

normal cells (Boucher *et al.*, 1986).

The sodium channels present in airway and many other epithelial cells vary in their exact characteristics, but are generally distinguished by their sensitivity, as a family, to the inhibitor amiloride. These channels are otherwise classified based on biophysical properties including channel kinetics, cation selectivity, and pharmacology (Benos *et al.*, 1995). Sodium channel complexes have been biochemically purified from a number of cells or tissues such as bovine renal collecting tubules, toad urinary bladder, and renal A6 cells (Benos *et al.*, 1995). An amiloride-sensitive epithelial sodium channel (rENaC) has also been cloned from rat distal colon (Canessa *et al.*, 1993; Canessa *et al.*, 1994). This channel consists of three homologous subunits (α, β, and γ), of which the α subunit alone is sufficient for channel activity when expressed in planar lipid bilayers (Canessa *et al.*, 1993; Canessa *et al.*, 1994). The β and γ subunits cannot individually act as sodium channels, but may be involved in conferring regulatory properties to the channel formed by the association of the three subunits (Benos *et al.*, 1995; Ismailov *et al.*, 1997). While the main conductance state of the immunopurified bovine renal channel is 39 pS, DTT treatment reduces this conductance state to 13 pS, which is consistent with the single-channel conductance reported for rENaC (Benos *et al.*, 1997). The suggestion has been made, therefore, that α,β,γ-rENaC is the core conductance unit of the native sodium channel protein complex (Benos *et al.*, 1997)

The introduction of CFTR into MDCK cells transfected with the cloned rat epithelial sodium channel causes a decrease in Na^+ current through these channels; furthermore, CFTR expression also alters the rENaC response to cAMP agonists in these cells. In the presence of CFTR, elevation of cAMP inhibits the Na^+ current, whereas in the absence of functional CFTR, cAMP agonists stimulate the Na^+ current (Stutts *et al.*, 1995b). The sodium hyperabsorption in CF airways may therefore result from the lack of a natural inhibitor of ENaC activity, namely, the CFTR protein (Ismailov *et al.*, 1996; Stutts *et al.*, 1997).

The precise mechanism by which CFTR regulates the sodium channel is not

currently known. It seems clear that CFTR affects ENaC at the level of single-channel activity, rather than by increasing the number of channel units in the membrane (Stutts *et al.*, 1995b; Ismailov *et al.*, 1996). The possibility exists that CFTR interacts directly with ENaC to inhibit channel activity: when immunopurified CFTR is coincoporated into planar lipid bilayers with rENaC, the single-channel activity of rENaC is decreased compared to rENaC activity in the absence of CFTR (Ismailov *et al.*, 1996). CFTR has no effect on the channel formed by α-rENaC alone (Ismailov *et al.*, 1997), but appears to require the presence of the β and the γ subunits to exert its inhibitory influence on sodium channel activity (Ismailov *et al.*, 1997).

In addition, CFTR may influence sodium channel activity through other protein intermediaries. Many recent reports have implicated cytoskeletal elements in the control of epithelial ion transport (Mills & Mandel, 1994). In particular, short actin filaments have been shown to increase the activity of both CFTR and ENaC; monomeric actin or actin in the longer filamentous (F-actin) form have no such effect (Cantiello, 1995; Prat *et al.*, 1995; Berdiev *et al.*, 1996).

Short actin filaments are thought to confer PKA responsiveness to ENaC; only those actin filaments phosphorylated by PK A can stimulate sodium channel activity (Prat *et al.*, 1993; Cantiello, 1995; Berdiev *et al.*, 1996). Short actin polymers (trimers or tetramers) have been shown to physically interact with the α subunit of rENaC (Ismailov *et al.*, 1997). The actin-mediated sensitivity of rENaC to PK A is inhibited by the presence of functional CFTR (Ismailov *et al.*, 1996; Mall *et al.*, 1996). Nonfunctional CFTR, such as the ΔF508 mutant (Mall *et al.*, 1996) or the G551D mutant (Ismailov *et al.*, 1996), does not inhibit ENaC activity.

Hence, in airway cells expressing both normal CFTR and these sodium channels, activation of PKA may cause the simultaneous activation of chloride conductance and inhibition of sodium transport. In cells either lacking CFTR or containing nonfunctional mutants, PKA activation would have the opposite effect: chloride secretion would not be elicited, but sodium transport would be upregulated.

In summary, CFTR may exert regulatory control over amiloride-sensitive

sodium channels in at least two ways: first, through direct protein-protein associations, and second, through interactions mediated by the actin cytoskeleton.

2.3.1.4.3 CFTR as a Potential Transporter of Other Substances

2.3.1.4.3.1 CFTR-associated Bicarbonate Secretion

The ability of the small intestine to secrete bicarbonate ions into the lumen plays an essential role in stabilising the pH of this extracellular compartment. This is particularly important in the duodenum, which is continually exposed to acidic material entering the gut from the stomach. While much of this duodenal bicarbonate secretion is electroneutral and occurs through the action of the Cl^-/HCO_3^- exhanger, and some bicarbonate transport can be measured through the paracellular pathway, a significant portion occurs electrogenically (Allen *et al.*, 1993). cAMP agonists have been shown to stimulate electrogenic bicarbonate transport in the duodenum (Allen *et al.*, 1993) and in airway, biliary, and pancreatic epithelia (Illek *et al.*, 1997).

Because cAMP-regulated bicarbonate secretion is also defective (Smith & Welsh, 1992) in addition to the defective chloride conductance in CF airway epithelia, an interest was taken in the potential role of CFTR in the bicarbonate secretory process. Under basal conditions, airway epithelia secrete H^+ into the mucosal fluid; cAMP agonists prevent excess acidification of the airway fluid, presumably by stimulating bicarbonate secretion (Smith & Welsh, 1993). 3T3 fibroblasts or C127i mouse mammary carcinoma cells transfected with ΔF508 CFTR, and mock-transfected cells, cannot modify their internal pH in response to an acid exposure (Poulsen *et al.*, 1994). 3T3 or C127i cells transfected with wild-type CFTR, on the other hand, show an ability to recover rapidly from this acidification (Poulsen *et al.*, 1994). The CFTR-like channel which appeared after transfection of these cells exhibits a bicarbonate permeability, albeit with a quarter of its permeability to Cl^- (Poulsen *et al.*, 1994).

Several studies have been carried out using the CF knockout mouse to investigate the effects of defective CFTR on bicarbonate secretion in the small intestine. The consensus is that not only are basal levels of bicarbonate secretion lower in the

duodena of *cftr* (-/-) mice, but the bicarbonate secretory response to cAMP agonists is significantly diminished compared to that of normal, *cftr* (+/+) littermates (Grubb, 1995; Hogan *et al.*, 1997a; Hogan *et al.*, 1997b). This is consistent with the earlier observations that the cAMP-induced bicarbonate secretory response is defective in CF airway epithelia (Smith & Welsh, 1992) and pancreatic epithelia (Welsh *et al.*, 1995).

Bicarbonate secretion can be stimulated by agents which increase intracellular cGMP production as well as by those which increase the production of cAMP. Guanylin, for example, increases electrogenic bicarbonate secretion as well as chloride secretion by rat duodenal mucosa, and this bicarbonate secretion can be inhibited by CFTR channel blockers (Guba *et al.*, 1996). Interestingly, while cGMP agonists appear to increase intestinal chloride and bicarbonate secretion in a roughly equivalent fashion, cAMP agonists have a much greater effect on chloride secretion than on bicarbonate secretion (Guba *et al.*, 1996; Field, 1996).

The physiological mediator of this bicarbonate transport is still under debate. The demonstrated ability of CFTR to conduct bicarbonate ions, though with a much lower selectivity than for chloride, the absence of cAMP-induced bicarbonate secretion in CF epithelia, and the sensitivity of the cGMP- and cAMP-stimulated bicarbonate secretion to known CFTR inhibitors such as NPPB (Guba *et al.*, 1996), DPC, and glybenclamide (Illek *et al.*, 1997) are cited as evidence that CFTR is directly responsible for bicarbonate transport (Field, 1996; Guba *et al.*, 1996; Illek *et al.*, 1997). Other investigators agree that while functional CFTR is required for normal bicarbonate secretion, it is not the sole candidate for the electrogenic pathway (Grubb, 1995). The ORCC, for example, is more permeable than CFTR to bicarbonate ions (Tabcharani *et al.*, 1989), and its regulation is also defective in cystic fibrosis. Hence, while CFTR is definitely associated with this conductive pathway, the evidence is not yet conclusive that CFTR acts as the physiological mediator of epithelial bicarbonate secretion.

2.3.1.4.3.2 CFTR-associated ATP transport

The ability of extracellular nucleotides to stimulate ion transport has been

documented in a variety of epithelial cell types (Knowles *et al.*, 1991; Dho *et al.*, 1992). The ORCC can be activated by extracellular ATP, for example, and it has been suggested that the regulatory effect of CFTR on the ORCC may involve autocoid activation by this extracellular nucleotide (Stutts *et al.*, 1992; Schwiebert *et al.*, 1995b). This led to the speculation that CFTR might either activate a distinct ATP transport mechanism, or itself serve as an ATP-permeable pathway (Schwiebert *et al.*, 1995b).

This distinction is a critical and, currently, a controversial one. A number of investigators have published reports consistent with a model in which the CFTR molecule acts as the mediator of ATP transport across the cell membrane. Others have been unable to reproduce these findings, or have observed phenomena which directly contradict this model.

In C127i cells, a cAMP-activated whole-cell ATP current was observed in cells transfected with wild-type CFTR, but not in mock-transfected cells (Reisin *et al.*, 1994). This current exhibited a pattern of sensitivity to chloride channel blockers similar to that of the whole-cell chloride current attributable to CFTR. Measurements of the reversal potential indicated that the ATP^- anion was the charge carrier, and no current was observed when ATP^- was replaced with SO_4^{2-} (Reisin *et al.*, 1994)

In the CF airway epithelial cell line IB3-1, release of $^{32}\gamma$-ATP was only observed when CFTR-transfected cells were stimulated with cAMP agonists; no such release was seen in mock-transfected or unstimulated cells (Schwiebert *et al.*, 1995b). The CFTR blocker glybenclamide inhibited ATP release. Both research groups furthermore demonstrated, in excised membrane patches, single-channel ATP currents through channels with biophysical properties very similar to those of CFTR (Reisin *et al.*, 1994; Schwiebert *et al.*, 1995b).

Other groups were unable to demonstrate ATP transport through CFTR in a number of experimental systems. ATP permeability could not be shown in human sweat duct or the Calu-3 lung cell line, both of which express CFTR endogenously (Reddy *et al.*, 1996), nor was it observed in Chinese hamster ovary cells stably transfected with CFTR (Grygorczyk *et al.*, 1996; Reddy *et al.*, 1996). This last study

directly contradicts another report in which CFTR-associated ATP channels were observed in the plasma membrane and outer nuclear membrane of CFTR-transfected CHO cells (Pasyk & Foskett, 1997). Two independent groups of investigators further demonstrated that ATP was not transported through purified CFTR reconstituted into planar lipid bilayers (Li *et al.*, 1996a; Reddy *et al.*, 1996).

Because electrophysiological studies may not be sensitive enough to record very small fluxes of ATP across membranes, ATP-release luminometry assays have also been performed on a variety of cell types. Again, contradictory results have been reported. In assays performed on CFTR-transfected C127i cells, CFTR expression was shown to correlate with cAMP-stimulated ATP transport into the extracelluar medium (Prat *et al.*, 1996). Similar results were reported in T84 cells, and this release of ATP could be blocked by the introduction of antisense CFTR oligonucleotides (Abraham *et al.*, 1997). Other investigators have reported that under similar conditions T84 cells, as well as a number of other epithelial cell types, were not observed to release ATP into the extracellular medium following cAMP stimulation, though these cells often released ATP when subjected to mild mechanical stimuli (Grygorczyk & Hanrahan, 1997a; Grygorczyk & Hanrahan, 1997b).

No consensus currently exists, therefore, concerning whether CFTR itself mediates ATP transport from the intracellular to the extracellular environment, or whether it simply activates a closely associated ATP channel. Variations in experimental conditions may account for some of the conflicting observations, but a number of reports remain to be reconciled before a workable model can be constructed to explain the relationship between CFTR and ATP transport.

2.3.1.5 Regulation of CFTR Activity

The activity of CFTR is relatively low in resting cells. In cells which have been stimulated by chemical or hormonal agonists, however, chloride currents attributable to CFTR increase greatly. Under baseline conditions, therefore, channel activity of CFTR must be under tight regulatory control, and this activity must have the ability to change

depending upon the physiological status of the cell. The CFTR protein must consequently contain mechanisms whereby it can respond to stimuli with a change in channel activity.

2.3.1.5.1 Regulation by Serine/threonine Kinases

2.3.1.5.1.1 Regulation by cAMP-dependent Protein Kinase (PKA)

Physiological and pharmacological agents that are classified as cAMP agonists act by stimulating the production of the cytosolic second messenger cAMP through the activation of membrane-associated adenylate cyclase. cAMP initiates a cascade of intracellular events by converting protein kinase A from its inactive to its active form; the activated kinase then modifies the activity of substrate proteins by phosphorylating them on serine or threonine residues.

cAMP agonists cause an increase in the whole-cell chloride-selective current in cells expressing CFTR (Tabcharani *et al.*, 1990; Cliff and Frizzell, 1990; Kartner *et al.*, 1991). The addition of the purified catalytic subunit of PKA and ATP to excised membrane patches from CFTR-transfected cells also results in the activation of a chloride-selective single-channel current (Berger *et al.*, 1991; Tabcharani *et al.*, 1991). Moreover, purified recombinant CFTR reconstituted into planar lipid bilayers is also activated by the addition of PKA and ATP (Bear *et al.*, 1992). This chloride conductance can be reversed by the removal of cAMP or by the addition of the Walsh peptide, an inhibitor of PKA (Tabcharani *et al.*, 1991). Clearly, therefore, activation of PKA results in increased CFTR activity.

The protein kinase-A mediated activation of CFTR may be direct, through phosphorylation of CFTR itself, or indirect, through phosphorylation of other proteins which then act upon CFTR. Because CFTR contains ten classic consensus sites for PKA phosphorylation (Riordan *et al.*, 1989), it seemed likely that CFTR was a direct target for kinase activity. Nine of these ten PKA consensus sites are contained within the R region of the CFTR protein (Riordan *et al.*, 1989), and phosphorylation of CFTR by PKA has been demonstrated both *in vitro* (Gregory *et al.*, 1990; Cheng *et al.*, 1991; Picciotto *et al.*, 1992) and *in vivo* (Cheng *et al.*, 1991; Tabcharani *et al.*, 1991; Picciotto

et al., 1992).

Specific residues have been identified as targets of PKA-mediated phosphorylation. In general, the results of the *in vitro* and *in vivo* phosphorylation assays correlate, in that all of the residues reported to be phosphorylated *in vivo* are also phosphorylated *in vitro* (Cheng *et al.*, 1991; Picciotto *et al.*, 1992; Dulhanty & Riordan, 1994). For example, Cheng and coworkers reported the phosphorylation of seven serine residues *in vitro*, and four of those seven were phosphorylated *in vivo*. (Cheng *et al.*, 1991). All of the serine residues which are phosphorylated *in vivo* are contained within the R region (Cheng *et al.*, 1991; Picciotto *et al.*, 1992).

The relative contribution of each phosphoserine to CFTR activation is a controversial subject. Point mutation studies in which individual serine residues were converted to non-phosphoacceptor alanines have reported conflicting results. The substitution of any single serine has little effect on CFTR activity in response to PKA (Cheng *et al.*, 1991), and while, originally, triple and quadruple mutants were thought to show a greatly reduced response (Cheng *et al.*, 1991), later studies showed that these mutants still retain the ability to respond to PKA (Chang *et al.*, 1993). In fact, CFTR still remains responsive to PKA even after mutagenesis of all ten potential phosphoacceptor sites from serine to alanine (Chang *et al.*, 1993).

Because these ten consensus sites are categorised as "classical" or "strong" sites for PKA phosphorylation, the proposal was made that CFTR may contain additional cryptic or weaker consensus sequences that can also be phosphorylated by PKA, and that these sites might be responsible for the CFTR response in the absence of the ten classic sites (Chang *et al.*, 1993). The 10SA mutant retains approximately 25% of the responsiveness of the wild-type CFTR, even though phosphorylation of this mutant by PKA could not originally be detected *in vitro* (Chang *et al.*, 1993). The investigators concluded that the PKA-responsiveness of the 10SA mutant must be mediated by a previously undetected phosphoacceptor serine. In fact, the use of systems expressing large amounts of CFTR allowed these investigators to confirm that serine 753 is phosphorylated in the 10SA mutant, and that conversion of this serine to an alanine

residue eliminates most of the remaining PKA-responsiveness of the 10SA mutant (Seibert *et al.*, 1995). The relevance of Ser-753 phosphorylation to the activity of wild-type CFTR in physiological situations, however, has not been determined.

An alternative possibility is that phosphorylation may have different effects on different serine residues. A recent report suggests that phosphorylation has a stimulatory effect on some serine residues in the R domain and an inhibitory effect on others, and that the final activity level of CFTR in response to PKA might therefore be an additive one (Riordan, 1994; Wilkinson *et al.*, 1997).

Intriguingly, construction of a mutant in which the R domain is deleted (CFTRΔR) results in a constitutively active channel (Rich *et al.*, 1991), unlike wild-type CFTR, which requires a cAMP-mediated stimulus to attain maximal activity. Based on these findings, the proposal was made that the R domain normally inhibits channel activity, and that phosphorylation of sites within the R domain might either contribute to a conformational change which allows ion movement through the CFTR transmembrane pore (Rich *et al.*, 1991), or that phosphorylation might increase the electrostatic repulsive forces between the R domain and some other part of the protein, presumably the mouth of the ion channel (Rich *et al.*, 1991; Fuller & Benos, 1992).

In fact, PKA-mediated phosphorylation of shark and human R domain polypeptides has been shown to cause a decrease in the amount of α-helix in the protein, with a corresponding increase in the random coil content as measured by circular dichroism spectroscopy (Dulhanty & Riordan, 1994). The investigators suggested that these findings support the notion of a conformational change causing a shift of the R domain, following phosphorylation, to expose the mouth of the CFTR ion channel and allow ion passage (Dulhanty & Riordan, 1994). Hence, an analogy has been made to the ball-and-chain model of the Shaker potassium channel, in that the R domain may act as a tethered "ball" which can reversibly plug the CFTR ion pore (Fuller & Benos, 1992; Rich *et al.*, 1993).

2.3.1.5.1.2 Regulation by Protein Kinase C (PKC)

In addition to the consensus sites for PKA, the CFTR R domain also contains seven consensus sequences for phosphorylation by protein kinase C (PKC); additional PKC consensus sites are found outside the regulatory region (Riordan *et al.*, 1989). The sites within the R domain can be phosphorylated by PKC *in vitro* (Picciotto *et al.*, 1992; Berger *et al.*, 1993) and *in vivo* (Picciotto *et al.*, 1992). However, while the consensus sites outside the R region can be phosphorylated *in vitro*, they do not appear to be phosphorylated *in vivo* (Picciotto *et al.*, 1992).

Protein kinase C is activated when certain agonists increase the cell membrane turnover of phosphatidylinositol phosphate, an event which results in the generation of inositol trisphosphate (IP_3) and diacylglycerol (DAG) (Nishizuka, 1995). DAG is directly responsible for PKC activation, while IP_3 stimulates the release of calcium from intracellular stores (Nishizuka, 1995). Protein kinase C can also be activated by phorbol esters, a class of tumor-promoting agents that includes phorbol 12-myristate 13-acetate (PMA) and phorbol 12, 13-dibutyrate (PDB) [Newton, 1995].

The role of PKC in the regulation of CFTR is less clear than the role of PKA. In 3T3 cells (Berger *et al.*, 1993) and CHO cells (Tabcharani *et al.*, 1991) transfected with CFTR, the addition of PKC to excised membrane patches stimulated CFTR channel activity. In both cases, however, the magnitude of the current activated by PKC was much smaller than that activated by PKA (Tabcharani *et al.*, 1991; Berger *et al.*, 1993). Furthermore, while one group reported that PKA-stimulated currents were higher in the presence than in the absence of PKC (Tabcharani *et al.*, 1991), the other group found no such effect (Berger *et al.*, 1993).

A more recent report suggests that in the CHO expression system, constituitive phosphorylation of CFTR by PKC may be required for acute PKA activation of the channel (Jia *et al.*, 1997). CFTR channel activity normally decreases over time after exposure of excised patches to PKA, a phenomenon referred to as channel rundown; this rundown could be reversed in these studies by the addition of PKC to the bath solution. In addition, pretreatment of the cells with the PKC inhibitor chelerythrine

prevented the activation of CFTR by PKA. The investigators suggested that CFTR channel rundown is due in part to the progressive dephosphorylation of PKC sites, and furthermore that the initial stimulus of CFTR by PKA requires a basal level of CFTR phosphorylation by PKC (Jia *et al.*, 1997). These results are consistent with the findings of several other investigators, but whether this phenomenon can be reproduced in other systems remains to be studied.

In some epithelial cell types endogenously expressing CFTR, activation of PKC alone has a small but significant effect on chloride secretion, as well as exerting a synergistic effect on cAMP-stimulated secretion. Short-term activation of PKC by phorbol esters causes an increased chloride secretion in HT-29 cells (Vaandrager *et al.*, 1992; Bajnath *et al.*, 1993), though exposure to these tumor promoters for more than two hours tends to inhibit CFTR activity (Vaandrager *et al.*, 1992; Breuer *et al.*, 1993). The long-term inhibitory effects of PKC have been variously ascribed to PKC-mediated downregulation of basolateral potassium transport (Vaandrager *et al.*, 1992) and to increased CFTR proteolysis (Breuer *et al.*, 1993).

In T84 intestinal epithelial cells, most investigators report that activation of PKC by phorbol esters has no effect on chloride secretion (Warhurst *et al.*, 1991; Kachintorn *et al.*, 1992; Reenstra, 1993), though there are reports that exposure to PMA can stimulate an apical chloride conductive pathway (Lindeman & Chase, 1992). The predominant effect of PKC activation in this cell line appears to be the inhibition of cAMP-dependent chloride secretion (Trapnell *et al.*, 1991; Warhurst *et al.*, 1991; Shen *et al.*, 1993), though again, there are conflicting reports concerning this phenomenon (Reenstra, 1993). Various proposals have been made to explain this inhibitory effect, including PKC-mediated inhibition of basolateral potassium channels (Reenstra, 1993; Shen *et al.*, 1993), and, based on the PMA-induced decrease in CFTR message in these cells, downregulation of CFTR protein in the apical membrane (Trapnell *et al.*, 1991).

To summarize, while the effects of protein kinase A on CFTR activity are clearly always stimulatory regardless of the cell system under investigation, the consequences of protein kinase C activation are more obscure. A number of isoforms of

PKC have been identified (Newton, 1995; Nishizuka, 1995), with different biophysical properties and patterns of tissue expression. Future investigations will require more carefully defined experimental parameters in order to fully clarify the effects of this kinase on CFTR function.

2.3.1.5.1.3 Regulation by cGMP-dependent Protein Kinase (PKG)

A variety of enteropathogenic microorganisms can cause secretory diarrhea, a disease which can result in a potentially life-threatening loss of fluid and electrolytes. Certain *Escherichia coli* strains produce a heat-stable enterotoxin (STa), and this peptide is responsible in many cases for inducing the intestinal secretory response by stimulating the production of cGMP in mucosal epithelia (Vaandrager & De Jonge, 1994).

STa structurally resembles the endogenous intestinal epithelial peptide guanylin, and both molecules act through the same epithelial receptor to activate a membrane-associated form of guanylate cyclase (GC) (Forte & Hamra, 1996). At least two isoforms of guanylate cyclase exist, but the only form which can be detected in intestinal epithelia is the GC-C isoform (Jarchau *et al.*, 1994; Vaandrager & De Jonge, 1994). Activation of GC-C, like the activation of adenylate cyclase, results in an increased intracellular production of a cyclic nucleotide second messenger: in this case, cGMP. cGMP acts in a manner analogous to cAMP, activating cGMP-dependent protein kinases (PKG, also known as cGK), as well as regulating specific phosphodiesterases (Vaandrager & De Jonge, 1994) . There is also evidence that cGMP can gate certain channels through direct interactions with these proteins (Vaandrager & De Jonge, 1994).

The elevated cGMP levels evoked by STa binding correlate with large increases in chloride secretion into the intestinal lumen; salt and water absorptive activities are thought to be simultaneously inhibited (Vaandrager & De Jonge,1994). Functional CFTR is required for this chloride secretion, since in CF humans and CF knockout mice, the intestinal epithelium is unresponsive to cGMP analogues as well as to cAMP

(O'Loughlin *et al.*, 1991; Cuthbert *et al.*, 1994; Goldstein *et al.*, 1994; Vaandrager & De Jonge, 1994).

The mechanism by which this increase in cGMP activates CFTR is still under investigation. The secretory response to cGMP in T84 cells and in CaCo-2 intestinal epithelial cells has been ascribed to a cGMP-mediated activation of PKA, rather than to an activation of a cGMP-dependent kinase (Forte *et al.*, 1992; Tien *et al.*, 1993; Chao *et al.*, 1994; Cuthbert *et al.*, 1994), although one group of investigators reported that the secretory response in T84 cells was due solely to the activation of PKG (Lin *et al.*, 1992).

CFTR can be phosphorylated *in vitro* by PKG purified from bovine lung (Picciotto *et al.*, 1992; Berger *et al.*, 1993). In human airway epithelia, however, CFTR does not respond *in vivo* to cGMP agonists, nor can excised membrane patches containing CFTR be activated by the direct addition of cGMP and PKG (Berger *et al.*, 1993).

The different results obtained from airway and intestinal epithelia may be explained by a tissue-specific distribution of PKG isozymes. The enzyme purified from bovine lung is the type Iα isoform (Berger *et al.*, 1993), which is restricted in the intestine to smooth muscle cells of the lamina propria (Markert *et al.*, 1995). The isoform expressed in the epithelial layer of the small intestine and the proximal colon is the type II isoform (Jarchau *et al.*, 1994; Markert *et al.*, 1995). Importantly, PKG II mRNA cannot be detected in airway epithelia, nor can its presence be demonstrated in the intestinal epithelial cell lines T84, CaCo-2, or HT-29.cl19A (Jarchau *et al.*, 1994; Markert *et al.*, 1995). Outside the intestine, PKG II message has only been detected in brain and kidney (Jarchau *et al.*, 1994).

The phosphoacceptor sites on CFTR which are recognised by PKG I overlap with those recognised by PKA; the sites phosphorylated by PKG I *in vitro* are four of the sites phosphorylated by PKA (Picciotto *et al.*, 1992). Phosphopeptide mapping demonstrates that the pattern of *in vitro* phosphorylation of CFTR by PKG II is similar to that of PKG I, but with one significant exception. One peptide phosphorylated by

PKA and PKG II is not phosphorylated by PKG I (French *et al.*, 1995), though it is not clear which specific residue within the peptide is phosphorylated. Differential phosphorylation of the CFTR molecule by PKG I and PKG II may therefore account for the difference in the abilities of the two kinases to activate CFTR.

PKG II, but not PKG I, can activate CFTR in membrane patches excised from stably transfected 3T3 fibroblasts and the rat intestinal cell line IEC-CF7 (French *et al.*, 1995). Likewise, whereas coexpression of CFTR and PKG II in the rat intestinal cell line confers sensitivity to stimulation by cGMP agonists to these cells, coexpression of CFTR and PKG Iβ does not (Vaandrager *et al.*, 1997). These findings are consistent with a model in which the stimulation of chloride secretion in the intestine in response to cGMP agonists occurs only in the presence of functional GC-C, PKG II, and CFTR. The secretory response to cGMP agonists in cells lacking PKG II has been proposed to occur as a result of a cross-activation of PKA by very high levels of cGMP (Vaandrager & De Jonge, 1994; Markert *et al.*, 1995).

2.3.1.5.2 Regulation by Nucleotides

2.3.1.5.2.1 Regulation by Intracellular Nucleotides

The presence of ATP is an absolute requirement for CFTR activity. Phosphorylation of the R domain by protein kinase A, first of all, is necessary but not sufficient to open the channel (Anderson *et al.*, 1991; Bell & Quinton, 1993). Most investigators report that the channel will remain open after this PKA-mediated phosphorylation only as long as ATP or its hydrolyzable analogues remain present in the cell or bath solution (Anderson *et al.*, 1991; Nagel *et al.*, 1992).

The additional requirement for ATP over and above its role as a source of phosphorylation is related to its ability to interact with the two nucleotide binding domains of the CFTR molecule, labelled NBD1 and NBD2. Some debate initially arose as to whether hydrolysis necessarily accompanies ATP binding to these domains, due to the fact that some reports indicated that the nonhydrolyzable analogue 5'-adenosine (β,γ-imino) triphosphate (AMP-PNP) can stimulate CFTR activity in sweat glands

(Quinton & Reddy, 1992) and in T84 cells (Bell & Quinton, 1993). These investigators suggested that nonhydrolytic ATP binding to CFTR might provide a mechanism through which CFTR activation is linked to cellular energy levels in order to prevent depletion of energy reserves through excessive chloride secretion (Quinton & Reddy, 1992; Bell & Quinton, 1993; Riordan, 1994).

This activation by AMP-PNP appears to depend, however, on the phosphorylation status of CFTR. AMP-PNP only stimulates CFTR activity under conditions in which the protein has already been phosphorylated by PKA (Bell & Quinton, 1993; Hwang *et al.*, 1994; Carson *et al.*, 1995). Reports of CFTR activation by AMP-PNP can therefore be reconciled with reports that this nucleotide does not activate CFTR, since in these earlier studies the channel was not phosphorylated (Anderson *et al.*, 1991).

Mutation analysis and experiments with various nucleotide analogues support the hypothesis that the interaction of ATP with both nucleotide binding domains is part of the gating cycle of CFTR. Most of the reports concerning nucleotide interaction with CFTR are consistent with a cycle made up of four separate stages or states, all of which occur after the R domain has been phosphorylated by PKA (Gadsby & Nairn, 1994; Hwang *et al.*, 1994; Carson *et al.*, 1995). The first state occurs before the binding of ATP to either NBD, and in the second, ATP has bound to both domains. The channel remains closed in both of these states. Following ATP hydrolysis at the first NBD (NBD1), the CFTR channel opens. In the final state, hydrolysis of the second ATP at NBD2 promotes closing of the channel (Gadsby & Nairn, 1994; Hwang *et al.*, 1994; Carson *et al.*, 1995). Hydrolytic activity of CFTR was recently demonstrated, and shown to be dependent upon the presence of Mg^{2+} and phosphorylation of the CFTR protein, which confirms the proposed CFTR gating hypothesis (Li *et al.*, 1996b). While this model may not fully explain all of the observed aspects of CFTR gating, it serves as the best model of CFTR behaviour based on current knowledge.

2.3.1.5.2.2 Regulation by Extracellular Nucleotides

Extracellular nucleotides, and in particular ATP, are known to induce chloride secretion in a variety of epithelial and non-epithelial cell types (Dubyak & El-Moatassim, 1993). Epithelial cells which have been shown to respond to extracellular ATP include airway surface epithelium and tracheal submucosal glands, as well as pancreatic, biliary, and intestinal cell lines (Mason *et al.*, 1991; Dho *et al.*, 1992; McGill *et al.*, 1994; Yamaya *et al.*, 1996; Chan *et al.*, 1996).

Investigations of the effects of extracellular nucleotides are complicated not only by the existence of multiple receptor subtypes for these ligands, but also by the rapid metabolism of ATP in the extracellular medium by a variety of ectonucleotidases (Dubyak & El-Moatassim, 1993). Activity of these enzymes results in the generation of ADP, AMP, and adenosine, substances which have also been shown to have a stimulatory effect on chloride secretion in epithelial cells (Stutts *et al.*, 1992).

Transport of ATP from the intracellular to the extracellular environment may involve the exocytotic release of stored ATP (Dubyak & El-Moatassim, 1993) or the movement of ATP through specific plasma membrane transporters. As previously mentioned, some investigators have demonstrated permeability to ATP in membrane proteins such as P-glycoprotein and CFTR, though other reports are more consistent with an ATP transport pathway associated with, but distinct from, CFTR (Abraham *et al.*, 1993; Devidas & Guggino, 1997).

Purinergic receptors of at least five subtypes can be distinguished based on the relative affinities with which various nucleotides bind each receptor. The distribution of receptor subtypes varies according to cell type and tissue of origin (Dubyak & El-Moatassim, 1993), and purinergic receptors can be found on both apical and basolateral membranes in many epithelial cells (Knowles *et al.*, 1991; Mason *et al.*, 1991; Clarke & Boucher, 1992; Hwang *et al.*, 1996).

P_{2u} receptors appear to predominate in the apical membrane of airway surface epithelium and submucosal glands (Mason *et al.*, 1991; Yamaya *et al.*, 1996). These apical P_{2u} receptors stimulate chloride secretion via separate calcium-dependent and

calcium-independent pathways (Stutts *et al.*, 1994; Stutts *et al.*, 1995; Hwang *et al.*, 1996). Elevation of intracellular calcium activates an apical calcium-regulated chloride channel, while increased cAMP production is thought to activate CFTR and the ORCC (Stutts *et al.*, 1994; Stutts *et al.*, 1995a; Hwang *et al.*, 1996). Basolateral application of ATP in these cells activates a different population of purinergic receptors, and secretion in this case may be independent both of intracellular free calcium and cAMP levels, but appears to result from CFTR activity nonetheless (Hwang *et al.*, 1996).

CF airway epithelia respond differently to the application of extracellular ATP than do normal airway cells. Mucosal ATP or UTP stimulates chloride secretion in both normal and cystic fibrosis airway epithelial cells (Knowles *et al.*, 1991; Mason *et al.*, 1991; Clarke & Boucher, 1992; Stutts *et al.*, 1992), suggesting that apical purinergic receptors of the P_{2u} subtype cause the activation of a chloride secretory pathway distinct from CFTR. Since P_{2u} receptor activation is associated with an increase in intracellular free calcium levels, and because the calcium-regulated chloride secretory pathway is known to be preserved in CF (Widdicombe, 1986; Boucher *et al.*, 1989; Anderson & Welsh, 1991), it seems likely that ATP activates the calcium-regulated chloride channel in CF airway. Purinergic agonists are therefore included in the category of compounds which may have a possible therapeutic effect in CF (Clarke & Boucher, 1992; Collins, 1992).

Exposure of intestinal epithelial cells in monolayers to extracellular ATP also results in a chloride secretory response. The purinergic receptor profile of these cells remains a matter of debate, however. Both P_{2u} purinergic and A_2 adenosine receptors have been observed on the apical as well as the basolateral membranes of T84 cells, though there is some debate as to the relative contribution of each subtype to the net secretory response to extracellular ATP (Barrett *et al.*, 1989; Dho *et al.*, 1992; Stutts *et al.*, 1995a). A_2 adenosine receptors stimulate cAMP production, and because cAMP-activated chloride channels are thought to predominate in intestinal chloride secretion, it has been suggested that A_2 receptors rather than P_{2u} receptors may account for the majority of the intestinal secretory response to mucosal ATP (Stutts *et al.*, 1995a).

Luminal purinergic P_2 receptors have also been observed in other intestinal epithelial cell lines, including CaCo-2 cells and polarized HT29 subclones (Guo *et al.*, 1995; Merlin *et al.*, 1996; Inoue *et al.*, 1997). In CaCo-2 cells, both apical and basolateral membranes contain P_{2u} receptors; in addition, the apical membrane contains receptors specific for uridine, and ADP-specific receptors have been observed in the basolateral membrane (Inoue *et al.*, 1997). All of these receptors are linked to increases in intracellular calcium, which appears to result in chloride secretion due to CFTR activation subsequent to basolateral potassium efflux (Inoue *et al.*, 1997).

2.3.1.6 Function of CFTR in Intracellular Organelles

Not all investigators agree that the defect in plasma membrane CFTR activity alone can account for the entire spectrum of cellular abnormalities associated with cystic fibrosis. For example, CF mucus glycoproteins have elevated levels of fucosylation and sulfation and decreased sialylation compared to non-CF glycoconjugates (Wesley *et al.*, 1983; Cheng *et al.*, 1989; Dosanjh *et al.*, 1994; Zhang *et al.*, 1995). Defective sialylation has been proposed to increase the susceptibility of CF airway epithelia to infection by *Pseudomonas aeruginosa*, as these cells express high levels of a receptor for this organism, identified as asialoganglioside 1 (aGM_1) on their surfaces (Imundo *et al.*, 1995). *Pseudomonas* does not bind to the normally sialylated form of the molecule (GM_1), nor do nonpathogenic bacteria such as *Escherichia coli* bind to aGM_1, although *Staphylococcus aureus*, another common CF pathogen, does bind aGM_1 (Krivan *et al.*, 1988; Imundo *et al.*, 1995). Hence, the production of airway mucus that is simply dehydrated due to defective chloride secretion may not be sufficient to explain the prominence of infections by *Pseudomonas* and *Staphylococcus aureus* in the CF clinical profile.

Glycosylation of proteins and lipids occurs within the Golgi, and the addition of fucose, sulfate, and sialic acid in particular are thought to occur within the trans-Golgi network (Al-Awqati *et al.*, 1992). These reactions appear to be competitive for substrate, and are pH-dependent (Al-Awqati *et al.*, 1992; Barasch & Al-Awqati, 1993).

As such, the effectiveness of these enzymes depends strongly on the internal Golgi pH. The sialyltransferases have acid pH optima, for example, while the fucosyltransferases and sulfotransferases prefer to operate closer to neutral or slightly alkaline pH (Al-Awqati *et al.*, 1992). The internal pH of intracellular organelles is determined by the activity of an electrogenic vacuolar H^+ ATPase, and the acidification of these compartments has been shown to depend on chloride channels which operate in parallel to the proton pump in order to maintain the transmembrane potential (Glickman *et al.*, 1983; Mellman *et al.*, 1986).

An elegant proposal made some time ago linked this requirement for chloride channel activity with the abnormal glycosylation pattern seen in CF epithelia. One group of investigators reported that the pH of prelysosomes, endosomes, and the trans-Golgi network was significantly higher in CF nasal epithelia than in normal cells (Barasch *et al.*, 1991). They furthermore suggested that CFTR is responsible for the conductive chloride pathway in these organelles, and that the absence of CFTR activity generates this alkaline pH. High lumenal pH was predicted to favour activity of sulfotransferases over that of sialyltransferases, resulting in the production of hypersulfated, undersialylated glycoconjugates (Barasch *et al.*, 1991; Al-Awqati *et al.*, 1992; Barasch & Al-Awqati, 1993). These findings are consistent with most reports concerning the composition of CF mucus glycoproteins (Wesley *et al.*, 1983; Cheng *et al.*, 1989; Dosanjh *et al.*, 1994; Zhang *et al.*, 1995).

Functional CFTR has been demonstrated in the membranes of clathrin-coated vesicles (Bradbury *et al.*,1993), endoplasmic reticulum (Pasyk & Foskett, 1995), and endosomes (Lukacs *et al.*, 1992; Biwerski & Verkman, 1994; Dunn *et al.*, 1994). There is considerable debate, however, as to whether CFTR actually contributes to the acidification process in these compartments. Endosomal pH has been assessed in a number of CF and normal cells, for example, including 3T3 fibroblasts and CHO cells stably transfected with CFTR, T84 intestinal epithelia, and CFPAC-1 pancreatic CF cells, as well as a rescued transfectant of this cell line (Lukacs *et al.*, 1992; Biwerski & Verkman, 1994; Dunn *et al.*, 1994). In none of these studies was the pH of endosomes

from CFTR-expressing cells shown to significantly differ from the pH of endosomes purified from cells not expressing CFTR, or which expressed defective CFTR. Investigators concluded that while CFTR may be present and functional in these membranes, the control of endosomal pH occurs through CFTR-independent pathways.

Similar results were obtained in a study investigating the acidification of the trans-Golgi network. A technique was developed to measure the intra-Golgi pH in living cells, involving the selective fusion of liposomes containing fluorescent markers with the trans-Golgi space (Seksek *et al.*, 1996). The pH of the trans-Golgi was measured in 3T3 fibroblasts transfected with wild-type or with ΔF508 CFTR, and in untransfected fibroblasts, as well as in cells endogenously expressing CFTR such as the pulmonary Calu-3 cell line. Again, expression of CFTR did not significantly alter the pH of the trans-Golgi in any of the cells examined (Seksek *et al.*, 1996). Since those cells not endogenously expressing CFTR presumably contain an alternative vacuolar chloride conductance, these results are not entirely surprising.

They do, nonetheless, directly contradict the earlier reports of defective acidification in these intracellular organelles (Barasch *et al.*,1991). While methodological differences and the use of non-genetically matched cell types may account for some of these inconsistencies, CF glycosylation abnormalities may not currently be said with any degree of confidence to result from intracellular acidification defects.

The proposed permeability of CFTR to nucleotides might, however, explain the abnormal glycosylation patterns seen in CF. Functional CFTR has been demonstrated in the ER membrane of CFTR-expressing CHO cells (Pasyk & Foskett, 1995), and CFTR in this membrane has also been associated with a cAMP-regulated ATP permeability (Pasyk & Foskett, 1997), leading to the suggestion that CFTR might furthermore be associated with transport pathways for other nucleotides in the membranes of internal organelles. Specifically of interest is the transport of 3'-phosphoadenosine 5'-phosphosulfate (PAPS), which is synthesized in the cytoplasm and then transported into the Golgi lumen, where it serves as the sulfate donor for sulfation

reactions (Hirschburg & Snyder, 1987). CFTR in membrane patches excised from transfected CHO cells was reported to be permeable to PAPS as well as to ATP (Pasyk & Foskett, 1997). The authors speculated that CFTR might be involved in the regulation of PAPS concentration within the Golgi by serving as an alternative "leak" pathway, parallel to the PAPS transporter (Pasyk & Foskett, 1997). Absence of CFTR would consequently result in a higher intralumenal PAPS concentration, thereby favouring sulfation reactions at the expense of sialylation reactions.

A number of caveats apply to this hypothesis. The ATP permeability of CFTR is not an accepted phenomenon, and the PAPS permeability was only demonstrated in the presence of high nucleotide concentrations on both sides of the excised plasma membrane patches (Pasyk & Foskett, 1997). Furthermore, while the earlier report by these authors had concluded that ΔF508 CFTR is a functional chloride channel in the ER membrane, it is not known how the ΔF508 mutation might affect nucleotide permeability, if such a property exists. As well, this model does not offer an explanation for the absence of hypersulfation in cells that do not endogenously express CFTR. Consequently, while these preliminary findings may suggest an interesting avenue of investigation, further research will be required to clarify the association between CFTR and intracellular glycosylation.

The final intracellular role of CFTR under consideration concerns the insertion and retrieval of material from the plasma membrane via exocytosis and endocytosis. This hypothesis stems from reports that intracellular cAMP levels regulate membrane recycling (Bradbury *et al.*, 1992b; Sorscher *et al.*, 1992), and that vesicular chloride channels appear to be required for exocytosis of secretory granules (Gasser *et al.*, 1988).

The initial report directly linking CFTR activity with plasma membrane recycling demonstrated that cAMP agonists such as forskolin or CPT-cAMP inhibited endocytosis and stimulated exocytosis in CFPAC-1 cells transfected with functional CFTR, but not in mock-transfected or untransfected cells (Bradbury et al., 1992a). This led to the suggestion that cAMP not only stimulates chloride channel activity through activation of PKA, but also promotes the retention of chloride channel units at the cell

surface, thereby increasing whole-cell chloride conductance (Bradbury *et al.*, 1992a; Bradbury *et al.*, 1992b).

Similar results have been reported in a number of other cell types, including T84 intestinal epithelia (Prince *et al.*, 1994; Santos & Reenstra, 1994) and CFTR-transfected HeLa cells and 3T3 fibroblasts (Biwersi *et al.*, 1996; Howard *et al.*, 1996). As well, other investigators confirmed these results in transfected CFPAC-1 cells, and were further able to demonstrate that the inhibitory effects of forskolin on endocytosis are a property of wild type CFTR, but not the G551D mutant (Prince *et al.*, 1994).

The effects of cAMP on CFTR-associated membrane trafficking may be cell type-specific, however. cAMP does not appear to affect the rates of exocytosis or endocytosis in transfected CHO cells (Dho *et al.*, 1993), although CFTR is known to be present and functional in endosomes from such cells (Lukacs *et al.*, 1992). Likewise, forskolin stimulation of human tracheal 9HTEo- cells did not correlate with an inhibition of endocytosis (Santos & Reenstra, 1994). Furthermore, the inhibitory effects of forskolin on T84 cells could not be duplicated with CPT-cAMP by these investigators. This led to the proposal that both the regulation of CFTR activity and the control of plasma membrane recycling may be cAMP-dependent, but that they are distinct processes (Dho *et al.*, 1993; Santos & Reenstra, 1994).

In summary, while CFTR may be functional in the membranes of a number of intracellular organelles, a consensus has yet to be reached concerning the physiological importance of CFTR in these compartments, and what, if any, role defective intracellular CFTR might play in the development of CF-associated pathology.

2.3.2 Other Apical Chloride Channels

2.3.2.1 The Outwardly Rectifying Chloride Channel (ORCC)

As previously mentioned, the defective chloride conductance in cystic fibrosis was initially attributed to an outwardly rectifying channel first described in human tracheal epithelial cells (Welsh, 1986). This channel was cAMP-regulated in normal tissues, but did not respond to cAMP agonists in CF tissues (Welsh & Liedtke, 1986;

Schoumacher *et al.*, 1987; Li *et al.*, 1988). However, cloning of the CF gene allowed investigators to demonstrate that expression of this gene did not necessarily correlate with the presence of the ORCC in epithelial cells. Although the presence of the ORCC could be confirmed through depolarization-induced activation in T84, CaCo-2, PANC-1, and 9HTEo-/S cell lines, significant amounts of CFTR message could only be detected by reverse transcriptase PCR in T84 and CaCo-2 cells (Ward *et al.*, 1991). CFTR mRNA levels were extremely low in the pancreatic and tracheal cells compared to the intestinal cell lines.

Furthermore, the appearance of a small-conductance, linear, chloride-selective channel in cells transfected with the CF gene (Anderson *et al.*, 1991a; Kartner *et al.*, 1991) led investigators to question the relationship between the ORCC and the CF gene product. Expression of wild-type CFTR in CF bronchial epithelial cells restored the PKA sensitivity of the ORCC currents (Egan *et al.*, 1992), and while the CF knockout mouse contains potentially functional ORCC, these channels are unresponsive to PKA (Gabriel *et al.*, 1993). Thus, the presence of wild-type CFTR appears to be required for normal activation of ORCC in epithelial cells. This requirement has also been shown when the two proteins are co-incorporated into planar lipid bilayers and purified PKA and ATP are supplied in the bath solutions (Jovov *et al.*, 1995a).

Once separate identities had been established for CFTR and ORCC, it became clear that the two channels had distinct biophysical properties. The ORCC is anion-selective, with a halide permeability sequence of $I^- > Cl^- > Br^-$ (Halm & Frizzell, 1992). The current-voltage relationship of the ORCC is outwardly rectifying, meaning that the channel conducts more current at higher voltages in symmetrical chloride solutions (Welsh, 1986; Halm & Frizzell, 1992). The single-channel conductance of the ORCC is voltage-dependent: at depolarizing voltages, the channel conductance is 60-80 pS, while at hyperpolarizing voltages, the single-channel conductance ranges between 20 and 40 pS when measured in symmetrical chloride solutions (Frizzell & Halm, 1990; Halm & Frizzell, 1992). Also, as already mentioned, the ORCC can be activated both in normal and in cystic fibrosis cells by a sustained exposure to depolarizing voltages over +50

mV (Hwang *et al.*, 1989), which provides a convenient means of confirming the presence of the channel in excised membrane patches.

The pharmacological profile of the ORCC includes a sensitivity to anthracene-9-carboxylate and its derivatives such as DPC and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (Welsh, 1986; Frizzell & Halm, 1990). Unlike CFTR, the ORCC-mediated currents can be blocked by DIDS, and this distinction provides a useful method of distinguishing the two currents in whole-cell recordings (Schwiebert *et al.*, 1994).

A number of mechanisms appear to regulate the activation of the ORCC. In normal and in CF-complemented cells, but not in cystic fibrosis epithelial cells, the ORCC can be activated by exposure to cAMP agonists or to PKA and ATP (Welsh, 1986; Welsh & Liedtke, 1986; Schoumaker *et al.*, 1987; Li *et al.*, 1988; Egan *et al.*,1992).

ORCC are also regulated by PKC, although the response of the channel to PKC-mediated phosphorylation appears more complex than its response to PKA. Hwang and coworkers (1989) reported an activation of ORCC by PKC in normal airway epithelial cells, while ORCC from CF airway could not be activated by this kinase . Another group reported that this stimulatory effect of PKC was calcium-dependent; while PKC activated the ORCC at low (10 nM or less) Ca^{2+} levels, PKC actually inhibited ORCC activity at high (1μM) Ca^{2+} levels (Li *et al.*, 1989). The biphasic nature of this response might be due either to the action of distinct PKC isoforms with different calcium sensitivities, or to a calcium responsive element inherent in the ORCC itself.

For many years investigators using electrophysiological techniques could only observe the ORCC in excised membrane patches; not until modifications were made to existing protocols were ORCC currents obtained from whole-cell recordings. These modifications included recording at a more physiological temperature, rather than standard room temperature conditions, as well as increasing the MgATP concentration from 1 mM to 5 mM, which approximates intracellular concentrations of the nucleotide (Schwiebert *et al.*, 1994). The requirement for elevated ATP concentrations led

researchers to investigate the possibility of a regulatory role for ATP in ORCC activation. In fact, the presence of extracellular ATP as well as the presence of functional CFTR was found to be an absolute requirement for the activation of ORCC-mediated currents in these studies, in that trapping of extracellular ATP by hexokinase treatment abolished the cAMP sensitivity of the ORCC in normal airway epithelial cells (Schwiebert *et al.*, 1995b). These researchers also presented data that implicated the CFTR molecule itself in the transport of ATP across the plasma membrane from the interior of the cell (Schwiebert *et al.*, 1995b) though the role of CFTR as a possible ATP transporter remains controversial (Devidas & Guggino, 1997).

The model proposed to explain the regulatory relationship between CFTR and ORCC involves the transport of ATP into the extracellular space, either by CFTR or a CFTR-associated protein. This ATP then activates a purinergic receptor, most likely of the P_{2u} subtype, based upon the ability of various nucleotides to substitute for ATP. In turn, the purinergic receptor mediates the activation of the ORCC, either directly or through the actions of an associated G protein (Schwiebert *et al.*, 1995a). Pertussis toxin-sensitive G proteins can be copurified with an ORCC from bovine tracheal epithelia (Ismailov *et al.*, 1996), and $G\alpha_{i-2}$ have been implicated in the regulation of ORCC from human airway epithelia (Schwiebert *et al.*, 1995a), though purinergic receptor stimulation of this G protein has not yet been shown to activate the ORCC.

While some reports have shown that extracellular ATP alone can activate the ORCC (Schwiebert *et al.*, 1994; Schwiebert *et al.*, 1995b), other groups have not been able to reproduce these findings (Jovov *et al.*, 1995b), and have suggested that ATP transport may not be the only function mediated by CFTR in the regulation of the ORCC. Mutations in the CF gene result in a wide variation in the severity of the disease phenotype, and this variability cannot be predicted based solely on the location of the mutation within the CF gene (Welsh *et al.*, 1995). Because CFTR controls the ORCC as well as acting as a chloride channel itself, CF mutations which block both conductive pathways may result in a more severe clinical syndrome than mutations in which only one of the pathways is affected.

This hypothesis is supported by the finding that two CF mutations which are associated with varying clinical severity also differ in their ability to activate the ORCC. G551D CFTR is a mutant which causes severe pulmonary disease (Welsh *et al.*, 1995). When an ORCC purified from bovine tracheal epithelium was co-incorporated into a planar lipid bilayer with G551D CFTR, the addition of extracellular ATP could not activate the ORCC despite the presence of PKA (Jovov *et al.*, 1995b) in the bath solutions. Expressed in CF-lacking human airway cells, G551D retains the ability to form a small linear chloride channel, albeit with a lower conductance than wild-type CFTR, but cannot activate ORCC in these cells (Fulmer *et al.*, 1995). The A445E mutant, on the other hand, retains not only its inherent chloride channel activity, though with a lower conductance than wild-type, but also its capacity to activate ORCC (Fulmer *et al.*, 1995). A445E is associated with much milder pulmonary symptoms than G551D (Fulmer *et al.*, 1995; Welsh *et al.*, 1995), leading to the speculation that there may be a correlation between the ability of CFTR to act as a regulator of other channels and the severity of the associated disease phenotype (Fulmer *et al.*, 1995).

2.3.2.2 Calcium-Activated Chloride Conductances

Epithelial chloride secretion can also be induced by agents which act by increasing intracellular levels of free calcium. This category of secretagogues includes the muscarinic cholinergic agonist carbachol, the calcium ionophores A23187 and ionomycin, and hormones such as neurotensin, histamine, and substance P. Calcium may interact directly with effector proteins, or it may bind calmodulin, altering cell physiology through the activation of calmodulin-sensitive protein kinases (CaMK).

Calcium-regulated channels appear to have a more selective tissue distribution than cAMP-regulated chloride channels such as CFTR and the ORCC. These cAMP-dependent channels have been characterised in a variety of epithelial tissues including airway, intestine, pancreas, salivary and sweat glands. In each tissue, not only are the the biophysical properties of the channels fairly consistent, but increased chloride secretion in response to cAMP agonists can be attributed directly to chloride channel

activation.

The nature of the calcium-dependent chloride conductive pathway, on the other hand, is slightly more complex. In some cells or tissues, calcium agonists appear to activate a specific calcium-regulated chloride channel (Willumsen & Boucher, 1989; Cliff & Frizzell, 1990; Anderson & Welsh, 1991; Morris and Frizzell, 1993). In others, the chloride secretory event is secondary to the activation of basolateral potassium channels. Increased exit of potassium ions hyperpolarizes the cell, increasing thereby the driving force for apical chloride secretion, and it has been suggested that in these circumstances chloride exit occurs through already open channels such as CFTR (Anderson & Welsh, 1991; Anderson *et al.*, 1992; Barrett, 1993). In investigating calcium-regulated chloride secretion, then, investigators must be careful to distinguish between two potentially distinct exit pathways.

Cells which exhibit a calcium-regulated chloride channel include primary cultures of airway epithelia (Willumsen & Boucher, 1989; Anderson & Welsh, 1991; Wagner *et al.*, 1991), sweat gland secretory coil (Reddy & Bell, 1996; Reddy *et al.*, 1997) and salivary gland epithelia (Cook *et al.*, 1994; Arreola *et al.*, 1994). As mentioned previously, this calcium-dependent chloride current is preserved in CF airway (Frizzell *et al.*, 1986; Widdicombe, 1986; Boucher *et al.*, 1989; Anderson & Welsh, 1991; Wagner *et al.*, 1991) and in sweat gland epithelia (Sato & Sato, 1984; Reddy *et al.*, 1996). In fact, in CF knockout mice such as the *cftr*^{tm1Unc} strain, airway epithelial calcium-regulated chloride channel activity is upregulated, possibly as a protective mechanism against the loss of the cAMP-dependent pathway (Clarke *et al.*, 1994; Grubb *et al.*, 1994).

The role of calcium-regulated chloride channels in intestinal epithelia is more controversial. Several groups have reported calcium-activated single-channel chloride currents in intestinal cell lines such as T84 (Cliff & Frizzell, 1990; Anderson & Welsh, 1991; Worrell & Frizzell, 1991) and HT-29 (Morris & Frizzell, 1993) epithelia, but only in whole-cell recordings, obtained when undifferentiated cells are grown on impermeable supports. Under such conditions, the cells do not form polarized

monolayers. When cultured on surfaces that allow them to differentiate and become polarized, this calcium-activated current disappears if the basolateral membrane is permeabilized by exposure to nystatin, removing the calcium-dependent activation of potassium channels in that membrane (Anderson & Welsh, 1991). These investigators concluded that the calcium-regulated chloride channels seen in undifferentiated T84 cells are not expressed when the cells differentiate (Anderson & Welsh, 1991; Anderson *et al.*, 1992).

Other groups, however, have shown that calcium-regulated chloride currents are present in the intestinal epithelial HT-29 cells, both in the unpolarized parental line and a polarized clone of this line, HT-29Cl.19A (Vaandrager *et al.*, 1991; Morris *et al.*, 1993). The gating and kinetics of the chloride channel are similar in polarized and in unpolarized cells, though the density of channels present in the membrane appears to be considerably lower in polarized cells (Morris *et al.*, 1993; Morris & Frizzell, 1994). Consequently, at least some cell lines may retain this channel after attaining the fully differentiated state.

In intact normal intestinal epithelia, calcium agonists cause an increase in chloride secretion (Berschneider *et al.*, 1988; O'Loughlin *et al.*, 1991). Unlike CF airway epithelia, however, the calcium-dependent pathway is thought to be inactive in the intestinal epithelium of CF patients, since the chloride secretory response to calcium agonists is absent in the majority of CF small intestines studied (Berschneider *et al.*, 1988; Taylor *et al.*, 1988; O'Loughlin *et al.*, 1991).

Most strains of transgenic *cftr* (-/-) mice also show severe intestinal histopathology (Zeiher *et al.*, 1995; Grubb & Gabriel, 1997), resulting in early death of affected animals unless their diet is supplemented with a polyethylene glycol solution (Grubb, 1995). Electrophysiological studies examining the epithelial reponse to cAMP and calcium agonists in these mice have reported an absence of chloride secretion in response to both classes of agonists, consistent with a model in which CFTR is the sole mediator of intestinal chloride secretion (Clarke *et al.*, 1994; Cuthbert *et al.*, 1994).

An exception to this may be the $cftr^{tm1Unc}$ mouse. Most of these mice exhibit the

expected CF intestinal phenotype resulting from an absence of epithelial CFTR, and die either soon after birth (class I mice) or shortly after weaning (class II mice) (Rozmahel *et al.*, 1996). A subpopulation of these mice, however, denoted class III, survive well beyond the age of weaning. Although there are some pathological changes in the intestine of class III mice, they are not as severe as those seen in class I and class II mice. Furthermore, while CFTR-mediated chloride conductance is defective in the intestine of all three classes, the class III mice are alone in possessing an intestinal chloride-selective current which can be activated by calcium ionophores or by UTP (Rozmahel *et al.*, 1996; Wilschanski *et al.*, 1996). The presence of this conductive pathway has been shown to be related to the genetic background of the mice (Rozmahel *et al.*, 1996), though the mechanism through which this pathway becomes upregulated is unknown.

This phenotypic variability in CF mice may have implications for the intestinal effects of CF in humans. While early studies report that the response of CF intestinal epithelium to calcium agonists is typically absent (Berschneider *et al.*, 1988; Taylor *et al.*, 1988; O'Loughlin *et al.*, 1991; Veeze *et al.*, 1991), some groups have reported that in a minority of CF patients, jejunal or rectal epithelial biopsy samples show a small but significant secretory response to calcium agonists, though none to cAMP agonists (Taylor *et al.*, 1988; Veeze *et al.*, 1991). Few of the human CF intestinal studies have involved large numbers of experimental subjects, however, so the possible significance of these findings, or their relationship to the physiology of class III *cftr*^{tm1Hsc} mice, is currently unclear.

The properties of the calcium-regulated chloride current have been characterised to some extent in various epithelia. Unlike the current attributable to CFTR, the calcium-regulated current exhibits time- and voltage-dependence; this current activates in a time-dependent fashion at depolarizing voltages, and inactivates at hyperpolarizing voltages (Anderson *et al.*, 1992; Frizzell & Morris, 1994). The halide permeability sequence of this current, $I^- > Br^- > Cl^-$, also differs from that of CFTR, as does its sensitivity to chloride channel blockers (Cliff & Frizzell, 1990; Anderson & Welsh,

1991). The calcium-regulated chloride current is inhibited by DPC and by disulfonic stilbene derivatives such as DIDS. Furthermore, the effects of calcium agonists and cAMP agonists on chloride secretion are additive (Cliff & Frizzell, 1990; Anderson & Welsh, 1991; Frizzell & Morris, 1994), suggesting that the two classes of agonists stimulate different apical membrane chloride channels (Anderson *et al.*, 1992; Frizzell & Morris, 1994).

The single-channel basis of the calcium-regulated chloride conductance has been studied in some epithelia. The channel characterised in undifferentiated HT-29 cells has a single-channel conductance of 13 to 16 pS, with an outwardly rectified current-voltage relationship (Morris & Frizzell, 1993a). The properties of this intestinal epithelial channel are consistent with those of a 15 pS calcium-regulated chloride channel purified from bovine tracheal epithelium (Ran & Benos, 1991; Ran & Benos, 1992), though the precise relationship between the two has yet to be investigated. These properties are also similar to those of a calcium-activated chloride channel studied in rat parotid salivary gland (Arreola *et al.*, 1996), though again, any closer relationship has not been established.

Regulation of this channel activity appears to depend upon intracellular levels of free calcium. Agents such as histamine or carbachol increase chloride secretion by stimulating the release of calcium from intracellular stores; this is most often accomplished through the receptor-mediated activation of a phospholipase C isoform which stimulates the generation of inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], the messenger directly responsible for the release of stored calcium into the cytosol. Calcium chelators such as EGTA or BAPTA prevent the activation of chloride secretion in response to calcium agonists in airway and intestinal epithelia (Morris & Frizzell, 1993; Harris & Hanrahan, 1994).

At least some of the activating effects of calcium on this channel are mediated by the activation of the calmodulin-sensitive protein kinase II (CaMKII), both in intestinal epithelial cell lines (Worrell & Frizzell, 1991; Morris & Frizzell, 1993b) and in airway epithelia (Wagner *et al.*, 1991). Peptide inhibitors of this kinase prevent the

activation of the calcium-regulated chloride current (Wagner *et al.*, 1991; Morris & Frizzell, 1993b) without affecting either basolateral potassium channel activity or intracellular calcium mobilization (Morris & Frizzell, 1993b).

The chloride secretory response produced by calcium agonists is transient, unlike secretion in response to cAMP agonists, which is sustained as long as the stimulus remains present (Barrett, 1997). The absolute magnitude of the secretory response also correlates poorly with the levels of intracellular free calcium (Barrett, 1997). Because of these two findings, it has been suggested that in addition to initially activating stimulatory pathways, the actions of some calcium agonists may also include the subsequent generation of inhibitory signalling mechanisms (Kachintorn *et al.*, 1993; Vajanaphanich *et al.*, 1994).

The activation of phospholipase C stimulates the production of a number of other phosphoinositide metabolites as well as that of Ins(1,4,5)P_3 (Menniti *et al.*, 1993). Some calcium agonists, such as the muscarinic agonist carbachol, have been shown to increase the intracellular generation of one such metabolite, D-*myo*-inositol 3,4,5,6-tetrakisphosphate [Ins(3,4,5,6)P_4] (Kachintorn *et al.*, 1993). Furthermore, increases in Ins(3,4,5,6)P_4 levels parallel the inhibition of carbachol-induced chloride secretion in T84 and in CFPAC-1 cells (Kachintorn *et al.*, 1993; Vajanaphanich *et al.*, 1994; Ho *et al.*, 1997). The inhibitory effect on chloride secretion is specific to Ins(3,4,5,6)P_4 and not to any other phosphoinositide (Kachintorn *et al.*, 1993; Xie *et al.*, 1996).

The mechanism through which Ins(3,4,5,6)P_4 inhibits chloride secretion does not appear to involve the inhibition of basolateral potassium channels (Smitham *et al.*, 1996; Barrett, 1997). Ins(3,4,5,6)P_4 has been shown, however, to directly inhibit the activity of bovine tracheal calcium-activated chloride channels reconstituted into planar lipid bilayers (Ismailov *et al.*, 1996). Whether this channel serves as a physiological target of Ins(3,4,5,6)P_4 in epithelial cells has yet to be demonstrated.

Some calcium agonists appear to stimulate calcium-dependent chloride secretion through different pathways. Although both agents increase intracellular free calcium levels, the effects of histamine on chloride secretion are additive with those of carbachol

(Kachintorn *et al.*, 1993), and unlike carbachol, histamine does not stimulate the production of Ins(3,4,5,6)P_4 (Kachintorn *et al.*, 1993). Other agents, such as epidermal growth factor (EGF), do not themselves act as chloride secretagogues, but may inhibit previously activated calcium-dependent chloride secretion (Uribe *et al.*, 1996; Barrett, 1997). Also, as mentioned above, many of the purinergic receptor agonists act by increasing intracellular free calcium levels, and may activate calcium-regulated chloride channels in those cells possessing such channels (Knowles *et al.*, 1991; Mason *et al.*, 1991; Clarke & Boucher, 1992).

In summary, calcium-dependent chloride secretion provides an alternative to the cAMP-mediated pathway. In some epithelia such as sweat gland and airway, distinct calcium-regulated chloride channels have been shown to exist in the apical membrane, and this pathway is preserved in cystic fibrosis. In other tissues, especially intestinal epithelia, the calcium-dependent pathway appears to be defective in CF. The existence of separate apical calcium-regulated channels in native intestinal epithelia remains under investigation, though the existence of a CF mouse strain (*cftr*^{tm1Hsc}) in which this pathway is intact raises a number of interesting questions.

From the above discussion, it seems clear that there are multiple pathways controlling epithelial chloride secretion. To reiterate the aims of this project in their proper context, then, the purpose of this study was to identify, through a molecular biological approach, the gene and gene product responsible for one particular chloride conductance pathway previously characterised in the small intestine. It was further intended to investigate the expression of this gene in various porcine tissues. First, this would allow a correlation between the expression of the gene and the presence of the antigen recognised by an anti-chloride conductance antibody. And second, knowledge of the tissue expression of this gene would also permit a comparison of its tissue distribution with that of the other major chloride channels.

3.0 Cloning Strategies for the Isolation of PG33 cDNA

3.1 Introduction

Fluid secretion by the intestinal crypts is controlled primarily by transepithelial electrolyte transport, especially the secretion of chloride ions into the gut lumen via apical chloride channels. The clinical importance of chloride secretion is illustrated by the drastic pathological changes seen in various secretory diarrheas, including cholera, and in cystic fibrosis. Redundancy in chloride channel species may therefore act as a protective mechanism, providing epithelial cells with the ability to mobilize different protein populations in response to different hormonal or neural stimuli.

To date, a number of chloride channel species have been identified in the small intestine. This includes CFTR, the ORCC, and ClC-2, though the existence of calcium-regulated chloride channels in intact intestinal epithelium remains subject to debate. Whether these channels represent the full complement of chloride conductive pathways in the small intestine is also currently unknown.

Chloride conductance in the small intestine has been the focus of investigation in this laboratory for several years. The pig proved to be a useful experimental model for early *in situ* loop studies of fluid flux in response to various secretagogues (Forsyth & Gabriel, 1990; Uwiera *et al.*, 1992). Pigs have also been used as a source of material for the preparation of brush-border membrane vesicles from small intestinal epithelia (Forsyth & Gabriel, 1988; Forsyth & Gabriel, 1989a). These vesicles are useful as a means to study the activity of apical membrane proteins under controlled electrochemical conditions.

Studies using brush-border vesicles depend on the ability to selectively establish an electrical gradient across the vesicle membrane by manipulating the potassium and

chloride concentrations inside and outside of the vesicle during preparation (Forsyth & Gabriel, 1988; Forsyth & Gabriel, 1989a). In the presence of such gradients, addition of the potassium ionophore valinomycin immediately before an assay causes rapid entry of potassium into the vesicle, imposing an electrical gradient across the membrane. This in turn provides conditions favourable to the movement of chloride along its electrochemical gradient across the vesicle membrane. By comparing ^{36}Cl uptake under these conditions to uptake in the absence of a potassium gradient, chloride movement though non-conductive pathways such as the Cl^-/HCO_3^- exchanger can be excluded. As a result, electrogenic movement of chloride ions through strictly conductive pathways such as membrane channels can be calculated.

Under these experimental parameters, the characteristics of a chloride conductive pathway in porcine jejunal brush-border vesicles have been defined. The activity of this pathway appears to be dependent upon the buffer composition in which the vesicles are prepared; no conductance is observed when vesicles are prepared in imidazole-acetate buffers, but when the buffer contains N-2-hydroxylethylpiperazine-N'-2-ethanesulfonate (HEPES) and tetramethylammonium (TMA), an increase in chloride conductance can be demonstrated in the presence of a potassium gradient (Forsyth & Gabriel, 1988, Forsyth & Gabriel, 1989a). The kinetics of this conductance are saturable (Forsyth & Gabriel, 1988), and this pathway is only marginally sensitive to conventional chloride channel blockers such as anthracene-9-carboxylate or 4-acetamido-4'-isothiocyanolstilbene-2,2'-suphonic acid (SITS) (Forsyth & Gabriel, 1988).

The chloride conductance in HEPES-TMA-buffered vesicles can, however, be specifically inhibited by α-phenylcinnamate (Forsyth & Gabriel, 1989b), and this fact was used in a ligand protection assay to identify candidate proteins from porcine ileal brush-border vesicles. The inclusion of Cl^- and α-phenylcinnamate (α-PC) is thought to protect the chloride conductance protein when the remainder of the brush-border protein complement is labelled with phenyl isothiocyanate (PITC). Because the binding of α-PC to the conductance protein is uncompetitive, if fluorescein isothiocyanate (FITC) is

subsequently added to the preparation, the fluorescein group may then attach to this conductance protein and act as a reporter group for scanning fluorescence densitometry. This procedure identified fluorescein labelling on proteins from HEPES-TMA buffered vesicles in two molecular mass ranges, approximately 130 kDa and 23 kDa (Forsyth & Gabriel, 1991), though exact protein species could not be identified.

Parallel projects were designed to identify the protein species responsible for conductive chloride transport through the production of specific anti-chloride conductance antibodies (Gabriel *et al.*, 1992; Racette *et al.*, 1996). Because of the complex nature of the protein populations present in brush-border vesicles, and because of the technical difficulties associated with purification of membrane-associated proteins, a single protein species was not available for preparation of antigen for immunization. Instead, the entire complement of brush-border membrane protein was separated by SDS-PAGE, then subdivided into molecular size fractions and electroeluted. Each size fraction, five in total, served as the basis for an antigen group used to immunize Balb/c mice. The polyclonal antibodies raised in these mice were then tested for the ability to inhibit ^{36}Cl uptake in ileal brush-border membrane vesicles. Only antibodies from mice immunized with fraction I (molecular mass over 110 kDa) exhibited significant inhibitory ability in this assay (Gabriel *et al.*, 1992). This is consistent with the results of the FITC labelling studies, which implicated a protein of approximately 130 kDa in the chloride conductive process in these vesicles (Forsyth & Gabriel, 1991).

Possession of monoclonal antibodies offers certain advantages in the study of antibody-antigen interactions. The antigen fraction that had produced the anti-chloride conductance polyclonal antibodies consisted of a heterogenous group of proteins, and despite the elimination of protein species under 110 kDa from consideration, a significant number of candidates remained. Mice which had produced antibodies reacting positively in Western blots and which had inhibited conductive chloride uptake were therefore used as a source of spleen cells for the generation of monoclonal antibody-producing hybridoma cells (Kohler & Milstein, 1975).

Two hybridoma cell lines were eventually selected as antibody sources, based on the functional ability to inhibit ^{36}Cl uptake by porcine ileal apical membrane vesicles (Racette *et al.*, 1996). The hybridoma line IC2-4B2 produced an IgM-class immunoglobulin which was extremely effective at inhibiting ^{36}Cl uptake by vesicles, but which had a relatively low affinity for antigen as shown by dilution studies. The 2H6-21 hybridoma, on the other hand, was an IgG-producing cell line. This antibody had a higher affinity for antigen than the IgM monoclonal, but bound with a lower avidity in the functional assay (Racette *et al.*, 1996).

Repeated attempts to resolve the identity of the antigen through standard dot blot, Western, and Far-Western assays using these mAb were unsuccessful. The IgG monoclonal antibody has been shown, however, to immunoprecipitate a 90 kDa antigen from ^{35}S-labeled cultures of porcine jejunal enterocytes (IPEC-J2 cells) (Racette *et al.*, 1996). This species could only be immunoprecipitated when the cells were grown in the presence of tunicamycin, a compound which prevents the N-linked glycosylation of newly synthesized proteins (Racette *et al.*, 1996). The current working hypothesis is that the complex nature of the physical interactions in the brush-border-associated glycocalyx prevents antibody binding under any other conditions.

Still, further information was required about the nature of the protein responsible for chloride conductance in brush-border vesicles, though dot blot and immunoprecipitation studies had demonstrated that this antigen was not CFTR (Racette *et al.*, 1996). A porcine intestinal expression cDNA library had been obtained so that the mAb could be used to identify the gene encoding this antigen, in parallel with the protein characterization studies.

Expression libraries are constructed so that the foreign cDNA sequences are inserted into a phagemid or plasmid vector at a site under the control of an inducible promoter. Typically, this promoter is the *Escherichia coli lacZ* promoter, which controls the expression of mRNA coding for β-galactosidase. Induction of promoter function results in the production of mRNA coding for fusion proteins in which the initial amino acids of β-galactosidase are linked to the polypeptide sequences

initial amino acids of β-galactosidase are linked to the polypeptide sequences corresponding to the foreign cDNA. If the structural integrity of epitopes in this polypeptide is maintained, this fusion protein may react with specific antisera in a Western-type blot. The goal of this study was therefore to identify such a fusion protein, in the hopes of then obtaining the full-length transcript of the cDNA encoding the antigen recognised by the anti-chloride conductance mAb.

3.2 Materials and Methods

Custom porcine intestinal cDNA library: Invitrogen (kind gift of Dr. Brad Bosworth, University of Iowa). Human infant intestinal cDNA library: Invitrogen. Hybond-C nitrocellulose and Hybond-N+ nylon filters, Amersham. Alkaline phosphatase-conjugated anti-mouse IgG and anti-mouse IgM, Sigma. Digoxygenin-dUTP and alkaline phosphatase-conjugated anti-digoxygenin antibody, Boehringer-Mannheim. All other chemicals reagent or molecular biology grade. All primers custom synthesized by the University of Calgary Core DNA Services.

3.2.1 Expression Screening of the Porcine Intestinal cDNA Library

Approximately $5x10^2$cfu of the custom intestinal cDNA library were spread on sterile Hybond-C nitrocellulose filters overlaid onto 82-mm LB+ampicillin (100μg/mL) plates and grown overnight at 37° C. Sets of two duplicate filters were prepared from each master filter and grown on LB+amp plates in the presence of isopropylthio-β-D-galactopyranoside (IPTG) for 4 hours at 37°C. The colonies were then lysed by exposure to chloroform vapour for 15 min and the filters washed overnight in lysis buffer (100 mM Tris-HCl,pH 7.8, 150 mM NaCl, 5 mM $MgCl_2$, 1.5% BSA, 1μg/mL DNase I, 40 μg/mL lysozyme).

The resulting cellular debris was removed by two successive room temperature washes in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Tween 20 (TTBS). Filters were then blocked for 2-3 hours in TTBS containing 1% gelatin before addition of the primary antibody. This consisted of the 1C2 and 2H6 monoclonal antisera diluted 1:40 and 1:20 respectively in blocker. Incubation was carried out for 60 minutes before the

Binding of the mAb was detected after a 60-minute incubation with alkaline phosphatase-conjugated anti-mouse IgM and anti-mouse IgG, diluted 1:10 000 and 1:5000 respectively in blocker. Unbound secondary antibody was removed by three final 10 minute washes in blocker. Bound secondary antibody was visualised by developing the filters in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$ containing 225 µg/mL nitro-blue tetrazolium (NBT) and 175 µg/mL 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). Positive colonies were identified by the appearance of a purple precipitate in the same location on both duplicate filters, and the colour reaction was stopped by a water wash.

Immunoreactive colonies were picked from the master filter and inoculated into 100 mL cultures of 2YT broth containing 100 µg/mL ampicillin, then grown overnight at 37° C. Bacterial cells were pelleted for 20 minutes at 4000 x *g* and resuspended in 25 mM Tris-HCl, pH 8, 50 mM glucose, and 10 mM EDTA. Lysozyme was added to a final concentration of 2.5 mg/mL. Cells were lysed and chromosomal DNA and membrane-protein complexes precipitated by first adding freshly prepared 0.2 M NaOH containing 1% SDS, then precipitating on ice in 3 M potassium acetate/ 5 M acetic acid. The solution was centrifuged for 15 min at 2500 x *g* and the supernatant recovered by filtration. Nucleic acids were precipitated by the addition of 0.6 volumes of isopropanol and pelleted in a 10 minute spin at 2500 x *g*. The pellet was rinsed with 70% ethanol and resuspended, when dry, in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA (TE).

High molecular weight RNA species were eliminated by the addition of LiCl to a final concentration of 2.5 M followed by a 10 minute spin at 10 000 x *g*. The remaining DNA was recovered by precipitation in 1 volume isopropanol and another identical centrifugation. The resulting pellet was rinsed in 70% ethanol, dried, and resuspended in TE plus 20 µg/mL RNase A, followed by a 30 minute incubation at room temperature to degrade any remaining RNA.

Plasmid DNA was precipitated by the addition of NaCl and polyethylene glycol (PEG)-8000 to final concentrations of 0.8 M and 6.5% respectively. The resulting pellet was resuspended in TE; PEG-8000 and protein contaminants were removed by

successive extractions in phenol, phenol:chloroform, and chloroform. The purified plasmid DNA was precipitated by adding 0.25 volumes 10 M ammonium acetate and 2 volumes 95% ethanol and centrifuging, after a 10 minute incubation at room temperature, for 10 minutes at 13 000 x *g*. The pellet was washed in 70% ethanol and resuspended in sterile water for sequencing.

Sequencing of the cDNA inserts was by the Sanger dideoxy chain-termination method (Sanger *et al.*, 1977) using Sequenase v2.0 (USBiochemicals). One pmol of sequencing primer (either the flanking T7 promoter sequence as an antisense primer, or the Sp6 promoter sequence as a sense primer) was annealed to 10 μg of plasmid template in a reaction solution containing 40 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 50 mM NaCl for 5 minutes at room temperature. In the labelling reaction, 10 mM DTT, 10 μCi ^{35}S-dATP, 1.5 μM each dGTP, dCTP, and dTTP, and 2.6 units of T7 DNA polymerase (Sequenase v2.0) are added to the annealing reaction and incubated for 3 minutes on ice. Following this, the reaction is terminated by the addition of 33.3 μM each of dGTP, dATP, dTTP, and dCTP; as well, either ddGTP, ddATP, ddCTP, or ddTTP are added to a final concentration of 33.3 μM. The termination reaction is carried out for 3-5 minutes at 37°C, then stopped by the addition of 0.65 volumes of 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol in 95% formamide. Five μL each sequencing reaction was loaded onto a 5% Long Ranger polyacrylamide gel (J.T. Baker) containing 50% w/v urea. The reactions were electrophoresed in 0.6x TBE at 1700 V for 3-12 hours, then dried for one hour and exposed to X-ray film (Kodak) for 36 hours before developing.

3.2.2 Oligonucleotide Screening

3.2.2.1 Preparation of Digoxygenin-labeled Probes

Labeling of internal PG33 sequences with digoxygenin-dUTP was carried out by polymerase chain reaction (PCR) in a solution containing 20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100 containing 200 µM each dGTP, dATP, dCTP, 165 µM dTTP, 35 µm dig-dUTP, 2.5 units Taq polymerase,

and 25 pmol each sense and antisense primer. Amplification was complete after 30 rounds in which each cycle consisted of 94°C for 45 sec, 52°C for 45 sec, and 72°C for 1 min. For cDNA screening of the porcine intestinal library, the probe I sense primer was 911s [5' GGTCGATACAGCGTA] and the antisense primer was 241a [5' TTACGGTCCTTACCA 3']. For Southern blotting of clones, probe II was constructed using 896a [5' CTCCCAGAGCCCACACTTTTACG 3'] as an antisense primer and 1004s [5' ACAAGTTACCTTGGAATT 3'] as a sense primer. Probe III was constructed using PLas [5' GCATATGTCTGCAAACCACCTG 3'] as an antisense primer, and the Sp6 promoter sequence [5' GCTATTTAGGTGACACTATAG 3'] as a sense primer.

To eliminate cross-reactivity with vector sequences, probe III was digested with BstX I after digoxygenin labelling in a reaction solution containing 5 mM Tris-HCl, pH 7.9, 10 mM NaCl, 1 mM $MgCl_2$, and 0.1 M DTT overnight at 55°C. The digested DNA fragment were then separated by electrophoresis through a 0.8% low melting point agarose gel containing 0.4 μg/mL ethidium bromide. The gel was run in 1x TAE buffer, and the desired DNA band was cut out of the gel and eluted using a GeneClean kit (BIO 101).

3.2.2.2 cDNA Screening of the Porcine Intestinal Library

Master filters were prepared as described above. Duplicate filters were made using Hybond-N+ nylon filters and grown for 4 hours at 37°C. The duplicates were denatured by a 5 minute exposure to 1.5 M NaCl/ 0.5 M NaOH, neutralised for 5 minutes in 1.5 M NaCl/ 0.5 M Tris-HCl, pH 8, and DNA was crosslinked to the filters by a 30-second exposure to UV light.

Filters were prehybridised at 65°C in 5x S SC, 0.1% laurylsarcosine, 0.02% SDS plus 2% blocker (Boehringer-Mannheim), and hybridised overnight at 65°C in the same solution containing 15 ng/mL digoxygenin-labeled probe.

Unbound probe was removed by two 15-minute washes at room temperature, first in 2x SSC/ 0.1% SDS, then in 0.1x SSC/0.1% SDS. The filters were briefly equilibrated in buffer 1 (0.1M maleic acid, 150 mM NaCl, pH 7.5), then blocked for 30

minutes in buffer 1 containing 0.5% blocker. Alkaline phosphatase-conjugated anti-digoxygenin antibody (Boehringer-Mannheim) was diluted 1:5000 in blocker and incubated with the filters for another 30 minutes, then removed by three successive 10-minute washes in buffer 1. The colour reaction to visualise positive clones was carried out as described above. Plasmid purification and sequencing of cloned inserts was carried out as previously described.

3.2.3 Southern Blotting

Ten μL of the PCR product were electrophoresed on a 0.8% agarose gel containing 0.4 μg/mL ethidium bromide in 1x TBE buffer. The gel was denatured for one hour in 1.5 M NaCl, 0.5 M NaOH, then neutralised for one hour in 1.5 M NaCl, 0.5 M Tris-HCl (pH 8). Capillary transfer of nucleic acid to a Hybond N or Hybond N+ nylon membrane previously equilibrated in 20x SSC took place overnight in 20x SSC. The membrane was then rinsed briefly in 6x SSC, air dried, and the DNA was fixed to the membrane by a 30-second exposure to UV light. Membranes were prehybridised, hybridised, and developed as described above.

3.2.4 RNA Purification and Northern Blotting

Poly A+ RNA was purified directly from porcine parotid salivary gland using a QuickPrep Micro mRNA purification kit (Pharmacia). Postmortem tissue samples were taken from 15-20 kg weanling Yorkshire-Landrace mixed breed pigs (obtained from Prairie Swine Centre). 0.5 g tissue was homogenised for 30 seconds in 0.8 mL extraction buffer (5.5 M guanidium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarkosyl). Samples were diluted in 2 volumes of elution buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA), then cell debris was removed by a 1-minute centrifugation at 10 000 x *g*. Supernatant was resuspended in oligo(dT)-cellulose and mixed for 3 minutes on an orbital shaker. Samples were repelleted at 10 000 x *g* for 10 seconds, and the supernatant aspirated. Samples were washed five times with 1 mL high-salt buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 M NaCl), and twice with 1 mL low-salt buffer

(Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 M NaCl), before polyA+ RNA was eluted in low-salt buffer.

Twenty μg polyA+ parotid RNA was loaded onto a 1% agarose gel containing 6.6 % formaldehyde after denaturation of the sample for 15 minutes at 65°C in 1x MOPS running buffer (20 mM morpholinopropanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.0), 50% formamide, 2.2 M formaldehyde. Samples were electrophoresed in 1x MOPS running buffer, then transferred by capillary action to Hybond N+ nylon membranes overnight in 20x SSC. RNA was fixed to the membrane by baking for 2 hours at 80°C. Prehybridization took place at 65°C in 6x SSC, 5x Denhardt's solution (0.1% polyvinylpyrrolidone, 0.1% Ficoll 400, 0.1% bovine serum albumin), 100 μg/mL sheared denatured salmon sperm, 0.5% SDS. Hybridization occurred in the same solution at 65°C, with the addition of 15 ng/mL digoxygenin-labelled probe. Blots were washed and developed as described above.

3.2.5 PCR Screening of Porcine Intestinal Library

The gene-specific antisense primer 896a [5' CTCCCAGAGCCCACACTTTTACG 3'] was selected from near the 5' end of PG33c and used to directly amplify PG33 sequences from the pig intestinal library. The flanking Sp6 promoter sequence [5' GATTTAGGTGACACTATAG 3'] was used as a sense primer.

One μL of the porcine intestinal library containing 1 x 10^5 cfu/μL was amplified in a reaction solution containing 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 200 μM dNTPs, 5% DMSO, 20 pmol each primer, 2.5 units *Taq* polymerase, and 2.5 units *Taq* Extender (Stratagene) for 40 cycles at 94°C for 45 sec, 52°C for 45 sec, and 72°C for 1 minute 30 seconds.

Five 100-μL reactions were pooled and the mineral oil was removed by extraction with one volume of chloroform. The PCR products were precipitated with 0.1 volumes 3 M sodium acetate, pH 5.2, and 2.2 volumes 95% ethanol. The products were run on a 1% low melting point-agarose gel in TAE buffer, and the largest products

[size range between 800 and 1500 base pairs] were eluted from the gel using the GeneClean kit.

One ng of the eluted products was subjected to another 25 cycles of PCR amplification under the conditions described above. Five 100-μL reactions were chloroform-extracted, pooled, and ethanol precipitated. A 5' phosphate was added to the products after they were heated at 70°C for 5 minutes; the reaction contained 50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 5% PEG-8000, and 10 units T4 polynucleotide kinase (New England Biolabs). The reaction was carried out at 37°C for 30 minutes, then the enzyme was removed by phenol-chloroform extraction and ethanol precipitation with 2.2 volumes 95% ethanol.

3.2.5.1 Preparation of Vector DNA

The plasmid vector pcDNAII was digested with 20 units of EcoRV (NEB)in the presence of 10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, and 100 μg/mL BSA overnight at 37 °C. The enzyme was removed by phenol:chloroform extraction, and the cut vector was precipitated with 0.1 volume 3 M sodium acetate and 2.2 volumes 95% ethanol.

5' phosphates were removed by treatment with calf intestinal alkaline phosphatase (CIAP) (Gibco BRL) by incubating vector DNA at 50°C for 1 hour in the presence of 50 mM Tris-HCl, pH 8.5, 0.1 mM EDTA, and 1 unit/pmol DNA ends of the CIAP enzyme. At the end of the reaction, CIAP was removed by phenol:chloroform extraction. The DNA was ethanol precipitated and its concentration estimated by visualisation on an ethidium bromide-containing (0.33 μg/mL) 0.8% agarose gel.

3.2.5.2 Ligation and Transformation

1600 ng phosphorylated, blunt-ended PCR product was ligated into 50 ng EcoRV-cut pcDNAII in a reaction containing 50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 5% PEG-8000, and 1 unit T4 DNA ligase (Gibco BRL). The reaction was allowed to continue overnight at 16°C and the ligation was diluted 1:5 in

reaction was allowed to continue overnight at 16°C and the ligation was diluted 1:5 in TE before transformation.

Ten μL of the diluted ligation were tranformed into competent DH5α cells by incubating the DNA with the cells for 30 minutes on ice. The cells were then heat-shocked for 90 seconds at 42°C, left on ice for a further 90 seconds, and incubated with 2YT media in a shaking incubator at 37°C. After a sufficient time interval to allow expression of the ampicillin resistance phenotype, the cells were plated on 2YT+amp+ X-gal, and grown overnight at 37°C. In the presence of X-gal, bacteria transformed with recombinant plasmid DNA can be distinguished from nonrecombinants, as recombinant colonies are white while nonrecombinant colonies are blue.

Successfully transformed colonies were PCR screened using the 896a antisense and Sp6 sense primers as described above. Plasmid DNA from positive clones was purified and sequenced as previously described.

3.2.6 PCR Screening of Human Intestinal Library

$1x10^7$ cfu of a human infant intestinal library (Invitrogen) were PCR amplified for 40 cycles as described above using the 896a and Sp6 primers. A second, 25-cycle round was then performed on 1 μL of the products from the first round, this time using the antisense primer PLas [5' GCATATGTCTGCAAACCACCTG 3'] from near the 5' end of PG33d, and a different Sp6 sequence [cSp6: 5' AGGTGACACTATAGAATAC 3'] as a sense primer.

Five 100-μL reactions were pooled and 5'-phosphorylated as described above. 350 ng of the human PCR product were ligated into 25 ng of EcoRV-cut pcDNAII which had previously been treated with CIAP, then the ligated material was transformed as described previously into supercompetent DH5αF'I_q cells (Gibco BRL). Positive clones were selected for sequencing based on PCR screening of transformants with PLas and cSp6.

3.3 Results

The initial screening of the porcine intestinal expression library with the monoclonal antibodies 1C2 and 2H6 resulted in the selection of a cDNA clone which will subsequently be referred to as PG33. This clone reacted strongly with the antibodies in a Western blot assay, and restriction digest of the clone with Hind III and Xba I revealed that the size of the foreign insert was 943 base pairs (Figure 3.1, lane 2). The insert was sequenced and found to contain a continuous open reading frame in frame 2.

A 700-base pair probe constructed from internal sequences of PG33 was labelled with digoxygenin, and this probe was used to rescreen the porcine intestinal library. The first clone obtained was 1031 base pairs, approximately 100 base pairs larger than PG33 (Figure 3.1, lane 3). This clone is referred to as PG33b. Using the same techniques, a third clone was isolated. This clone contained the complete sequence of PG33 and PG33b plus approximately an additional 160 bases, making PG33c 1158 base pairs in size (Figure 3.1, lane 4). The open reading frame remained continuous throughout these two clones.

Attempts to obtain an estimate of transcript size by Northern analysis of small intestinal poly A+ RNA were unsuccessful. Poly A+ RNA was therefore purified from porcine parotid salivary gland to assess the size of the full-length PG33 transcript. Based on the Northern blot analysis, the size of the PG33 message is approximately 2.7 kb (Figure 3.2, lane 1).

Because of the apparent limitation on the size of the clones which can be identified by conventional cDNA screening, the next two clones were obtained using a PCR-based cloning strategy. This technique is designed to screen large numbers of recombinant plasmids rapidly and specifically for sequences of interest. The PCR products generated can then be subcloned directly into another vector for analysis. In this case, the inserts were subcloned into a vector derived from the pGEM lineage, pDK101, which had been cut before ligation with EcoRV.

The antisense primer 896a was selected from the sequence near the 5' end of

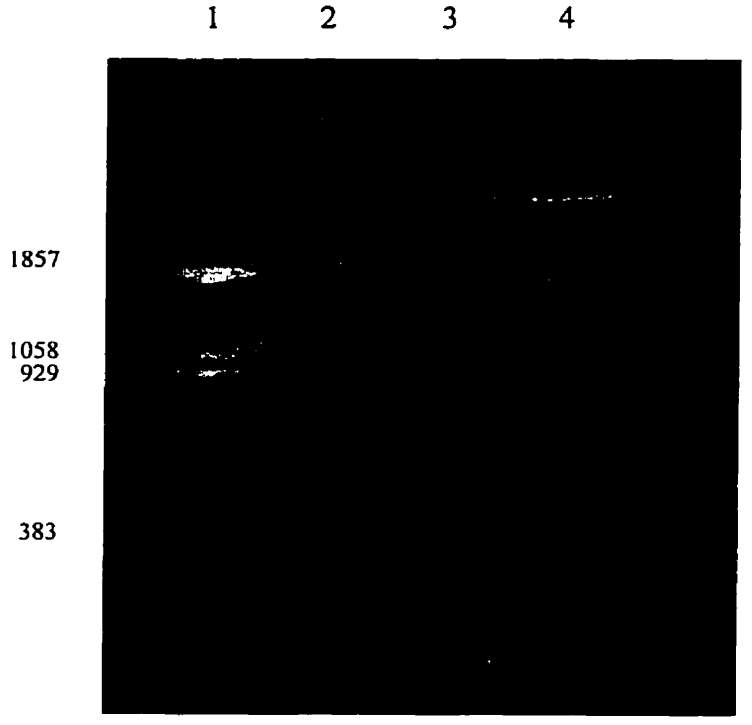


Figure 3.1. Hind III-XbaI digests of PG33 clones in pcDNA II. Lane 1: pBR322-BstN I standards (1857, 1058, 929, 383 bp). Lane 2: Hind III-Xba I digest of PG33. Lane 3: Hind III-Xba I digest of PG33b. Lane 4: Hind III-Xba I digest of PG33c.

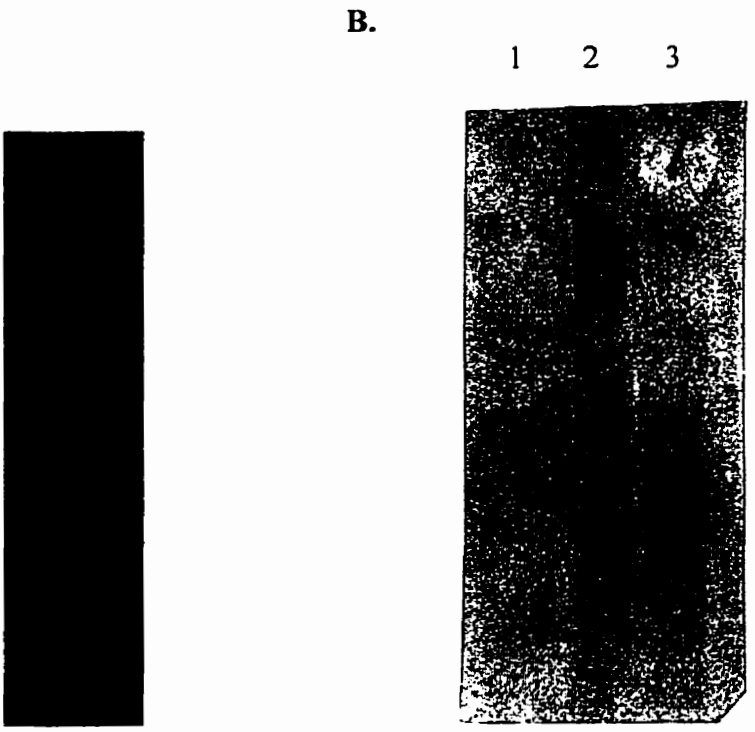


Figure 3.2. Northern blot demonstrating hybridization of PG33 probe I to porcine parotid salivary gland mRNA. Samples were electrophoresed on a denaturing gel, then transferred to Hybond N+ nylon membranes and hybridized with digoxygenin-labeled probe I. A: RNA standards (9.49, 7.46, 4.40, 2.37, 1.35, and 0.24 kb). B: Northern blot. Lane 1: 20 µg porcine parotid mRNA. Lane 2: Bam HI-cut PG33. Lane 3: unlabeled PCR product corresponding to the probe I sequences (positive control).

PG33c and used in conjunction with the Sp6 promoter primer from the pcDNAII vector to directly amplify the remaining PG33 sequences from the porcine intestinal library (see Figure 3.5 for schematic illustrating the overlap between PG33 clones). Since the library contains a heterogeneous population of cDNA inserts, this reaction resulted in the amplification of multiple PCR products of increasing size. The largest products (a population between 800 and 1500 base pairs in size) were selected for enrichment to increase the probability of obtaining the remainder of the PG33 transcript. This population was gel-eluted and then PCR-amplified a second time under the same reaction conditions, though with fewer total cycles, so that large amounts of product would be available for ligation.

This strategy resulted in the successful isolation of a fourth clone, 918 bp in size, which is referred to as PG33d. PG33d contains the sequence at the 5' end of PG33, and an additional 600 bp further 5' in the PG33 transcript (Figure 3.3A, lane 3). In a Southern blot of PG33c and PG33d, the PG33d clone can be seen to hybridize as strongly as the PG33c clone to a digoxygenin-labeled probe constructed from the 5' end of PG33c, whereas the plasmid vector pcDNA II did not react at all with the probe (Figure 3.3B). This PCR-based strategy is therefore a valid technique for selecting cDNA clones.

Because the sequence obtained through cloning of PG33d did not complete the full-length transcript of PG33, attempts were made to repeat this PCR-based strategy using the porcine intestinal library in order to obtain the sequences further 5' to PG33d. These attempts were unsuccessful; either the library does not contain full-length transcripts of this gene, or there are limitations on the ability of the PCR methodology to amplify this sequence from this particular template.

Instead, the antisense primer PLas, which had been designed from the 5' end of PG33d, was used to screen by PCR amplification a cDNA library constructed from human infant intestinal RNA. PCR amplification with PLas and the flanking Sp6 promoter primer as a sense primer generated a population of products of increasing size similar to those obtained during the amplification of PG33d. The largest of these PCR

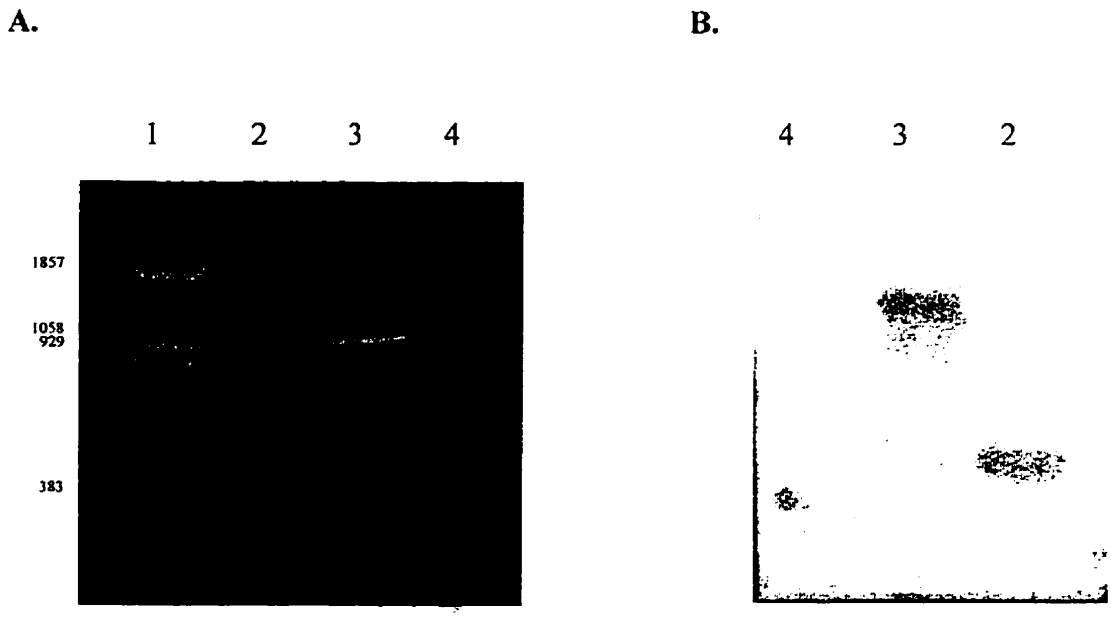


Figure 3.3. PCR-based cloning of PG33d from a porcine intestinal cDNA library. A: PCR amplification of clones PG33c and PG33d using the internal PG33 antisense primer 896a and the pcDNA II Sp6 promotor sense primer. Lane 1: pBR322-BstN I standards (1857, 1058, 929, 383 bp). Lane 2: PG33c amplified with 896a and Sp6. Lane 3: PG33d amplified with 896a and Sp6. Lane 4: pcDNA II vector amplified with 896a and Sp6 (negative control). B: Southern blot demonstrating cross-hybridization between PG33c and PG33d. DNA from gel in panel A was transferred to a nylon membrane and hybridized to digoxygenin-labeled probe II. Lane 4: pcDNA II vector amplified with 896a and Sp6. Lane 3: PG33d amplified with 896a and Sp6. Lane 2: PG33c amplified with 896a and Sp6.

products was subcloned into EcoRV-cut pDK101, and is referred to as PG33e. PG33e is 683 base pairs in size (Figure 3.4A, lane 3), and strongly cross-reacts in a Southern blot with a probe constructed from internal sequences from the porcine clone PG33d (Figure 3.4B).

The 5' end of the PG33d clone has a 188-base overlap with the 3' end of the PG33e clone, which allows a comparison to be made between the nucleotide and predicted amino acid sequences of the porcine and human PG33 genes. Nucleotide homology between the two genes in this region is 80% (Figure 3.6A). The predicted amino acid sequence has an 89% identity, which increases to 95% if conservative amino acid substitutions are included in the calculation (Figure 3.6B).

The full PG33 sequence obtained to date includes a 2322-base pair cDNA of which a 2040-base open reading frame encodes a 679-amino acid protein product (Figure 3.7). Hydrophobicity analysis of the predicted amino acid sequence indicates four potential transmembrane domains. Examination of the predicted amino acid sequence reveals the presence of 3 potential phosphorylation sites for protein kinase A and cGMP-dependent protein kinase, 15 potential phosphorylation sites for protein kinase C, 7 potential phosphorylation sites for CaMK II, and 8 potential sites for phosphorylation by casein kinase II. The predicted protein product also contains 6 consensus sites for N-linked glycosylation.

The cDNA sequence of PG33, when originally compared to known sequences, matched no DNA sequences contained in the GenBank database. Two recently identified genes, however, have been found to contain significant nucleotide and amino acid homology to the PG33 cDNA and predicted protein product. The first gene was identified in bovine tracheal epithelium and encodes the calcium-regulated chloride channel Ca-CC (Cunningham *et al.*, 1995). The PG33 cDNA cloned so far has a 60% nucleotide homology to the bovine chloride channel (Figure 3.8). Based on the deduced amino acid sequence, there is a 43% amino acid identity, increasing to 57% if conservative substitutions are also included (Figure 3.9).

The second gene with significant homology to the PG33 cDNA is lung-

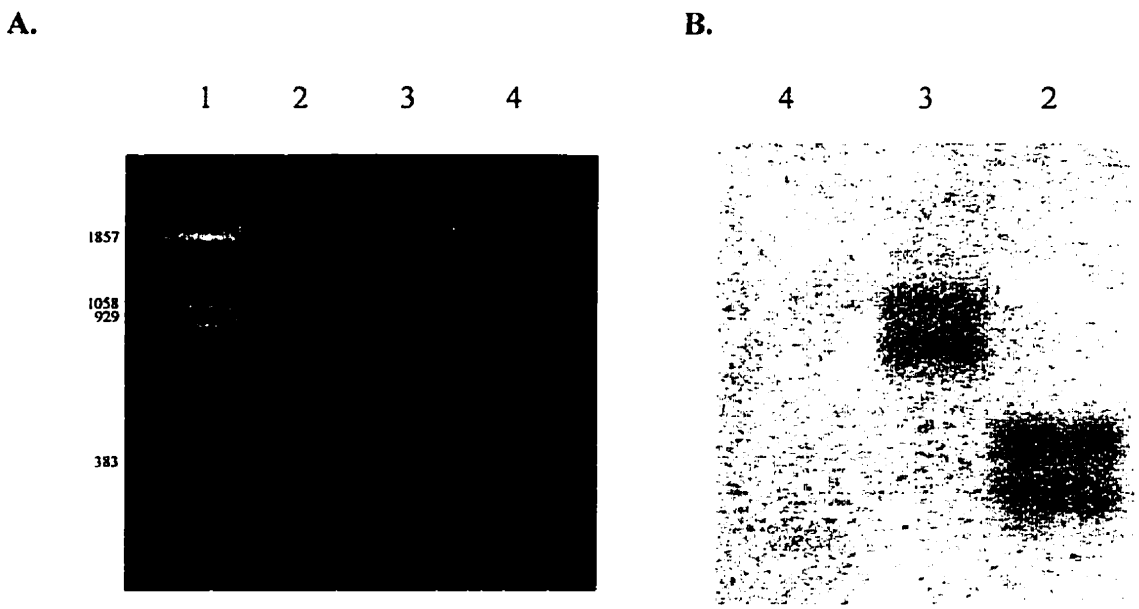


Figure 3.4. PCR-based cloning of PG33e from a human intestinal cDNA library. A: PCR amplification of clones PG33d and PG33e using the internal PG33 antisense primer PLas and the pcDNA II Sp6 promoter sense primer. Lane 1: pBR322-BstN I standards (1857, 1058, 929, 383 bp). Lane 2: PG33d amplified with PLas and Sp6. Lane 3: PG33e amplified with PLas and Sp6. Lane 4: pcDNA II vector amplified with PLas and Sp6 (negative control). B: Southern blot demonstrating cross-hybridization between PG33d and PG33e sequences. DNA from gel in panel A was transferred to a nylon membrane, then hybridized to digoxygenin-labeled probe III. Lane 4: pcDNA II vector amplified with PLas and Sp6. Lane 3: PG33e amplified with PLas and Sp6. Lane 2: PG33d amplified with PLas and Sp6.

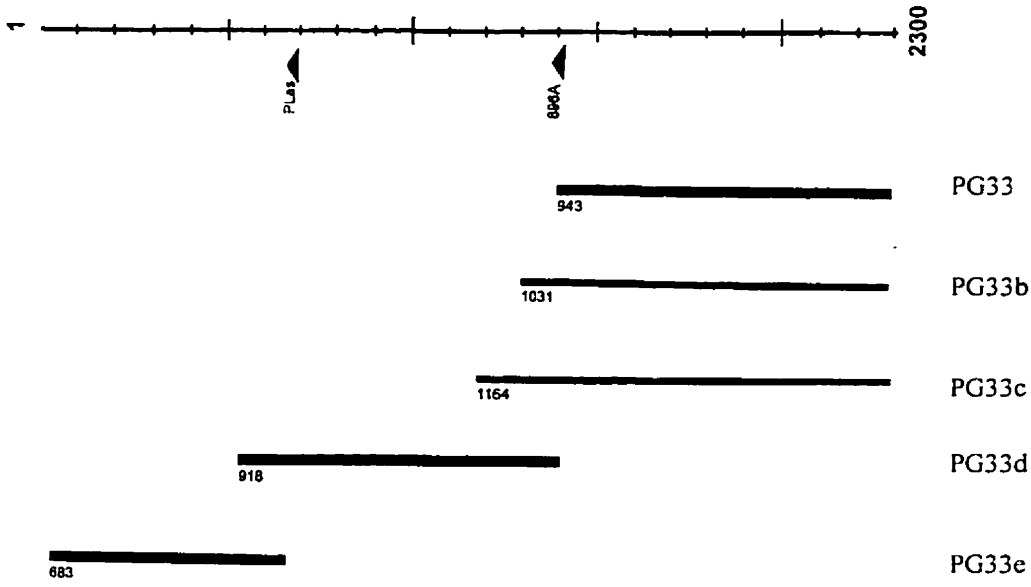


Figure 3.5. Schematic illustrating the overlapping arrangement of the PG33 clones. Arrows indicate the locations of the internal antisense primers 896a and PLas used to obtain the clones PG33d and PG33e.

A.

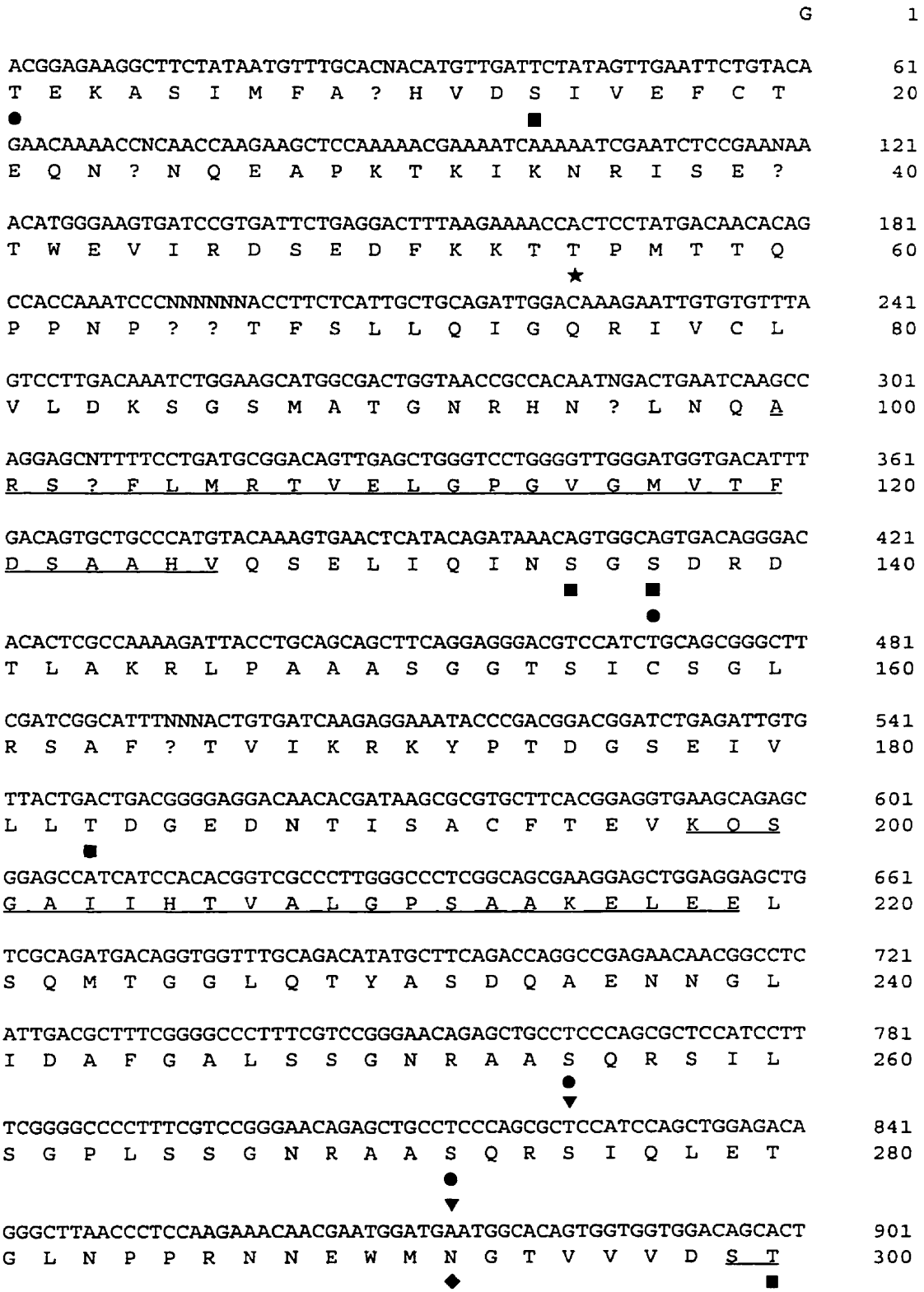
```
pig     TGTGATCAAGAGGAAATACCCGACGGACGGATCTGAGATTGTGTTACTGACTGACGGGGA
human   TGTGATTAGGAAGGAATATCCAACTGATGGATCTGAAATTGTGCTGCTGACGGATGGGGA
        ****** * ** * **** ** ** ** ******** ****** * ***** ** *****

pig     GGACAACACGATAAGCGCGTGCTTCACGGAGGTGAAGCAGAGCGGAGCCATCATCCACAC
human   AGACAACACTATAAGTGGGTGCTTTAACGAGGTCAAACAAAGTGGTGCCATCATCCACAC
         ******** ***** * ****** *  ***** ** ** ** ** **************

pig     GGTCGCCCTTGGGCCCTCGGCAGCGAAGGAGCTGGAGGAGCTGTCGCAGATGACAGGTGG
human   AGTCGCTTTGGGGCCCTCTGCAGCTCAAGAACTAGAGGAGCTGTCCAAAATGACAGGTGG
         *****  * ******** *****  * ** ** ***********  * ***********

pig     TTTGCAGA
human   TTTGCAGA
        ********
```

B.

```
pig   VIKRKYPTDGSEIVLLTDGEDNTISACFTEVKQSGAIIHTVALGPSAAKELEELSQMTGGLQ
human VIRKEYPTDGSEIVLLTDGEDNTISGCFNEVKQSGAIIHTVALGPSAAQELEELSKMTGGLQ
      **.. ********************.**.******************** ****** ******
```

Figure 3.6. Comparison of porcine PG33d sequences in the region overlapping with the human PG33e sequences. A: Porcine and human DNA sequence alignment. Asterisks under the sequence indicate nucleotide identity. B: Alignment of porcine and human predicted amino acid sequence. Asterisks under the sequence represent amino acid identity; dots indicate conservative amino acid substitutions.

Figure 3.7. Nucleotide sequence and predicted amino acid sequence of the translated PG33 cDNA. The full length of the current PG33 cDNA is 2322 base pairs, with a continuous open reading frame in the first 2041 bases encoding a 679-amino acid protein product. The four potential transmembrane domains are underlined. The protein sequence contains 6 consensus sequences for N-linked glycosylation (◆), 3 potential phosphorylation sites for cAMP- and cGMP-dependent proteins kinases (★), 7 potential sites for Ca^{2+}/calmodulin kinase II (▼), 8 potential sites for casein kinase II (■), and 15 potential sites for protein kinase C (●).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E Q N ? N Q E A P K T K I K N R I S E ? 40
ACATGGGAAGTGATCCGTGATTCTGAGGACTTTAAGAAAACCACTCCTATGACAACACAG 181
T W E V I R D S E D F K K T T P M T T Q 60
CCACCAAATCCCNNNNNNACCTTCTCATTGCTGCAGATTGGACAAAGAATTGTGTGTTTA 241
P P N P ? ? T F S L L Q I G Q R I V C L 80
GTCCTTGACAAATCTGGAAGCATGGCGACTGGTAACCGCCACAATNGACTGAATCAAGCC 301
V L D K S G S M A T G N R H N ? L N Q A 100
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R S ? F L M R T V E L G P G V G M V T F 120
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D S A A H V Q S E L I Q I N S G S D R D 140
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T L A K R L P A A A S G G T S I C S G L 160
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R S A F ? T V I K R K Y P T D G S E I V 180
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L L T D G E D N T I S A C F T E V K Q S 200
GGAGCCATCATCCACACGGTCGCCCTTGGGCCCTCGGCAGCGAAGGAGCTGGAGGAGCTG 661
G A I I H T V A L G P S A A K E L E E L 220
TCGCAGATGACAGGTGGTTTGCAGACATATGCTTCAGACCAGGCCGAGAACAACGGCCTC 721
S Q M T G G L Q T Y A S D Q A E N N G L 240
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I D A F G A L S S G N R A A S Q R S I L 260
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S G P L S S G N R A A S Q R S I Q L E T 280
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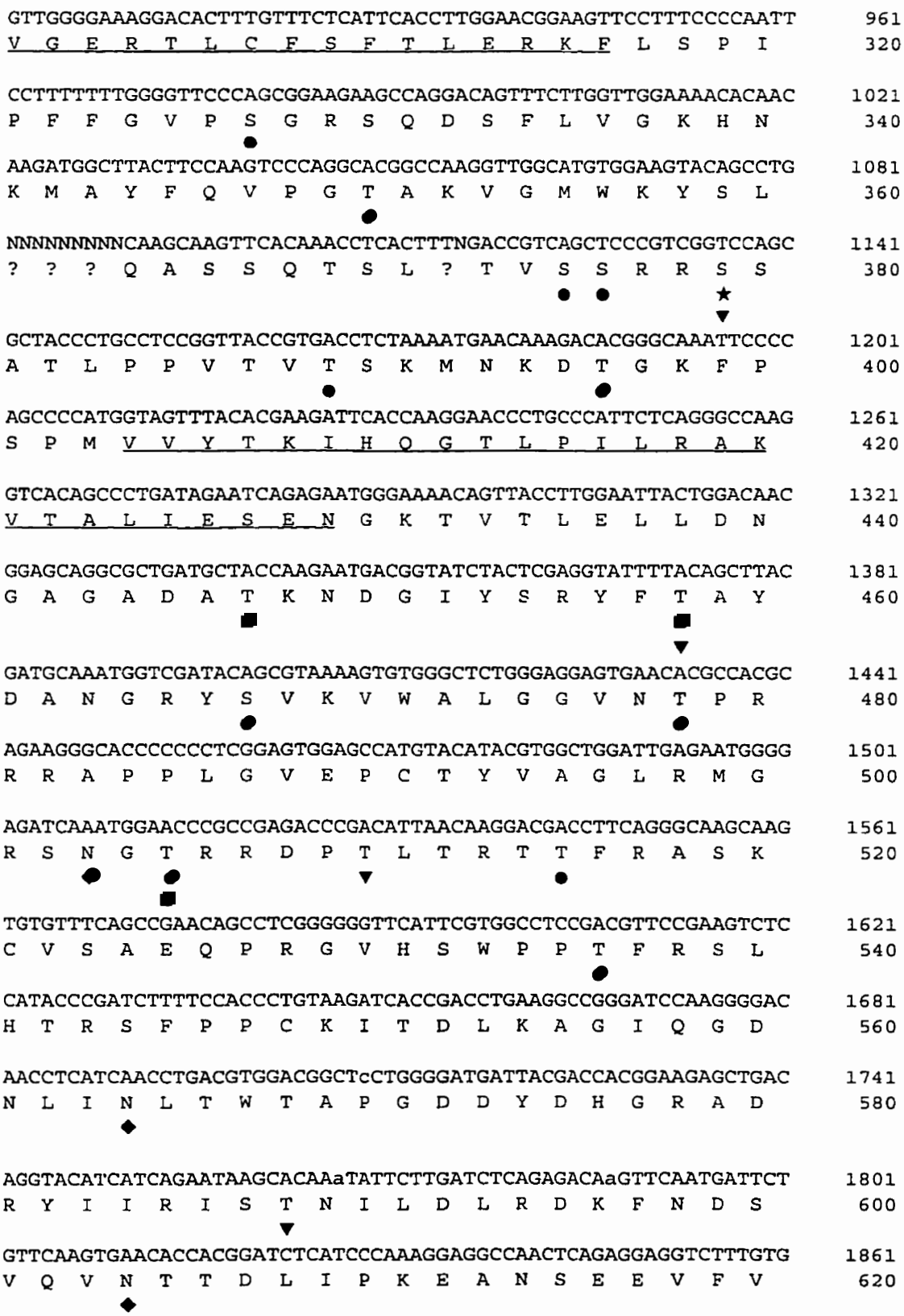
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P F F G V P S G R S Q D S F L V G K H N 340
AAGATGGCTTACTTCCAAGTCCCAGGCACGGCCAAGGTTGGCATGTGGAAGTACAGCCTG 1081
K M A Y F Q V P G T A K V G M W K Y S L 360
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? ? ? Q A S S Q T S L ? T V S S R R S S 380
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A T L P P V T V T S K M N K D T G K F P 400
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V T A L I E S E N G K T V T L E L L D N 440
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G A G A D A T K N D G I Y S R Y F T A Y 460
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D A N G R Y S V K V W A L G G V N T P R 480
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CATACCCGATCTTTTCCACCCTGTAAGATCACCGACCTGAAGGCCGGGATCCAAGGGGAC 1681
H T R S F P P C K I T D L K A G I Q G D 560
AACCTCATCAACCTGACGTGGACGGCTcCTGGGGATGATTACGACCACGGAAGAGCTGAC 1741
N L I N L T W T A P G D D Y D H G R A D 580
AGGTACATCATCAGAATAAGCACAAaTATTCTTGATCTCAGAGACAaGTTCAATGATTCT 1801
R Y I I R I S T N I L D L R D K F N D S 600
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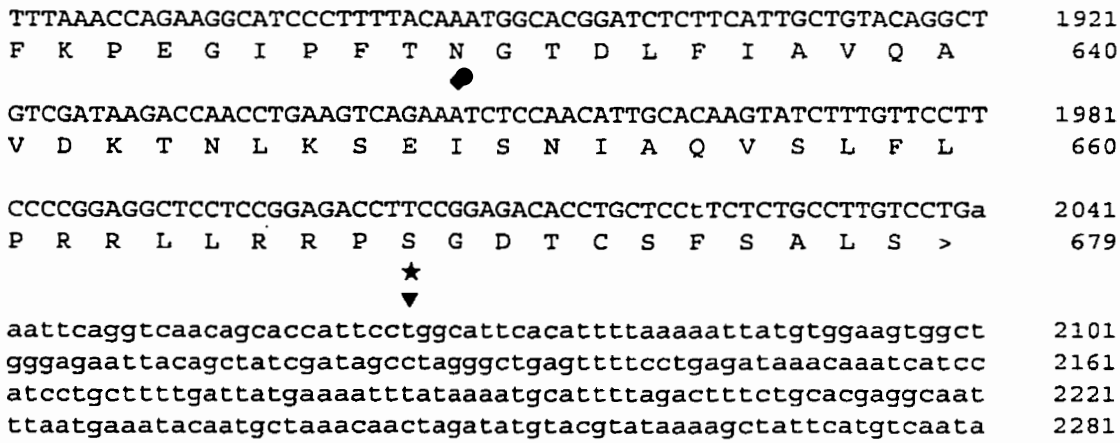
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V D K T N L K S E I S N I A Q V S L F L 660
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P R R L L R R P S G D T C S F S A L S > 679
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ttaatgaaatacaatgctaaacaactagatatgtacgtataaaagctattcatgtcaata 2281

Figure 3.8. Aligned nucleotide sequences of PG33, Ca-CC, and Lu-ECAM-1. Asterisks indicate nucleotides that are conserved between all three genes.

```
pg33          ------------------------------------------------------------
ca-cc         ----------------------------------------ATTGTAACATGCGCAA
lu-ecam-1     GGATTCCAGGGTCTCCAGCATTGCCTGAATCTGGATGTAGGTTTACTGTAACATGTGCAA

pg33          ------------------------------------------------------------
ca-cc         AAATGGTGCCTCGTCTGACTGTCATTCTGTTCCTAACTTTGCACCTCCTGCCTGGAATGA
lu-ecam-1     AAATGGTGCTCTGTCTGAATGTTATTCTGTTCCTAACTTTGCATCTCTTGCCTGGAATGA

pg33          ------------------------------------------------------------
ca-cc         AAAGTTCAATGGTAAATTTGATTAACAATGGATATGATGGCATTGTCATTGCAATTAACC
lu-ecam-1     AAAGTTCAATGGTAAATTTGATTAACAATGGGTATGATGGCATTGTCATTGCAATTAACC

pg33          ------------------------------------------------------------
ca-cc         CTAGTGTGCCAGAAGATGAAAAACTCATTCAAAACATAAAGGAAATGGTAACTGAAGCTT
lu-ecam-1     CCAGTGTGCCAGAAGATGAAAAACTCATTGAAAACATAAAGGAAATGGTAACTGAAGCTT

pg33          ------------------------------------------------------------
ca-cc         CTACTTACCTGTTTCATGCCACCAAACGAAGAGTTTATTTCAGGAATGTAAGCATTTTAA
lu-ecam-1     CTACTTACCTGTTTCATGCCACCAAACGAAGAGTTTATTTCAGGAATGTGAGCATTTTAA

pg33          ------------------------------------------------------------
ca-cc         TTCCAATGACGTGGAAGTCAAAATCTGAGTACTTAATGCCAAAACAAGAATCATATGACC
lu-ecam-1     TTCCAATGACCTGGAAATCAAAATCTGAGTACTTCATACCAAAACAAGAATCATATGACC

pg33          ------------------------------------------------------------
ca-cc         AGGCAGAAGTCATAGTTGCTAATCCTTACCTAAAACATGGAGATGATCCCTATACACTTC
lu-ecam-1     AGGCAGATGTCATAGTTGCTAATCCCTATCTAAAATATGGAGATGATCCCTATACACTTC

pg33          ------------------------------------------------------------
ca-cc         AATATGGAAGATGTGGAGAAAAAGGACAATATATACATTTTACTCCAAACTTCTTGCTGA
lu-ecam-1     AATATGGAAGGTGTGGAGAAAAAGGAAAATATATACATTTTACTCCAAACTTCTTGTTGA

pg33          ------------------------------------------------------------
ca-cc         CTAATAATTTGCCTATCTATGGGTCCCGAGGCAGAGCATTTGTCCATGAGTGGGCCCATC
lu-ecam-1     CTAATAATTTCCACATCTATGGGTCCCGAGGCAGAGTATTTGTCCATGAGTGGGCCCATC

pg33          ------------------------------------------------------------
ca-cc         TCCGATGGGGAATATTTGATGAGTATAACGGGGACCAGCCATTCTATATCTCCAGAAGGA
lu-ecam-1     TCCGCTGGGGAATATTTGATGAGTATAATGTGGACCAGCCATTCTATATTTCCAGAAAGA

pg33          ------------------------------------------------------------
ca-cc         ACACTATTGAAGCAACAAGATGTTCAACTCATATTACTGGTACTAATGTGATTGTCAA--
lu-ecam-1     ACACTATTGAAGCAACAAGATGTTCAACTCATATTACTGGTATTAATGTGGTTTTCAAGA

pg33          ------------------------------------------------------------
ca-cc         -ATGCCAGGGAGGCAGCTGTATAACAAGGCCATGCAGACGTGACTCACAGACAGGGCTGT
lu-ecam-1     AATGCCCTGGAGGCAGCTGTATAACAAGTCTATGCAGACGTGACTCACAGACAGGGCTGT

pg33          ------------------------------------GACGGAGAAGGCTTCTATAATGT
ca-cc         ATGAGGCAAAATGTACATTCATCCCAGAAAAATCCCAGACTGCAAGGGAGTCCATCATGT
lu-ecam-1     ATGAAGCAAAATGTACATTCCTTCCAAAAAAATCCCAGACTGCAAAGGAATCCATTATGT
                                                  *** *  * **  ** ** ****
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pg33          TTGCACNACATGTTGATTCTATAGTTGAATTCTGTACAGAACAAAACCNCAACCAAGAAG
ca-cc         TTATGCAAAGTCTCCATTCTGTGACTGAATTTTGTACAGAAAAAACACACAATGTGGAAG
lu-ecam-1     TTATGCCAAGTCTCCATTCTGTGACTGAATTTTGTACAGAAAAAACACACAATACAGAAG
              **   * *  * *  ***** *  ****** ********* ***  * ***    ****

pg33          CTCCAAAAACGAAAATCAAAAATCGAATCTCCGAANAAACATGGGAAGTGATCCGTGATT
ca-cc         CTCCAAACCTACAAAACAAAATGTGCAATGGCAAAAGCACATGGGATGTAATCATGAACT
lu-ecam-1     CTCCAAACCTACAAAACAAAATGTGCAATGGCAAAAGCACATGGGATGTAATCATGAACT
              *******     *** *****   * *    * **   ******** ** ***    * *

pg33          CTGAGGACTTTAAGAAAACCACTCCTATGACA---ACACAGCCACCAAATCCCNNNNNNA
ca-cc         CTACTGACTTTCAAAATACATCTCCCATGACAGAAATGAATCCACCGACTCAACCT---A
lu-ecam-1     CTGTTGACTTTCAGAATACATCTCCCATGACAGAAATGAATCCACCGACTCATCCT---A
              **   ****** * ** **  **** ******   *   * ***** * **        *

pg33          CCTTCTCATTGCTGCAGATTGGACAAAGAATTGTGTGTTTAGTCCTTGACAAATCTGGAA
ca-cc         CATTTTCATTGCTCAAGTCCAAACAGCGAGTAGTTTGTTTGGTACTTGATAAATCTGGAA
lu-ecam-1     CATTTTCATTGCTCAAGTCCAAACAGCGGGTAGTCTGTTTGGTACTTGATAAATCTGGAA
              * ** ********  **     ***  * * ** ***** ** ***** **********

pg33          GCATGGCGACTGGTAACCGCCACAATNGACTGAATCAAGCCAGGAGCNTTTTCCTGATGC
ca-cc         GCATGTCTTCAGAAGATCGTCTCTTTCGAATGAATCAAGCAGCAGAATTATTCTTGATTC
lu-ecam-1     GCATGTCTGCAGAAGACCGTCTCTTTCAAATGAATCAAGCAGCAGAACTATACTTGATTC
              ***** *  * *  * ** * *  *  * **********       * * * **** *

pg33          GGACAGTTGAGCTGGGTCCTGGGGTTGGGATGGTGACATTTGACAGTGCTGCCCATGTAC
ca-cc         AAATTATTGAAAAGGGATCCTTGGTTGGGATGGTTACATTTGACAGTGTTGCTGAAATCC
lu-ecam-1     AAGTTATTGAAAAGGGATCTTTAGTTGGGATGGTTACATTTGACAGTGTTGCTGAAATCC
                    ****   *    *    *********** ************* ***  *  * *

pg33          AAAGTGAACTCATACAGATAAACAGTGGCAGTGACAGGGACACACTCGCCAAAAGATTAC
ca-cc         GAAATAATCTAACAAAAATAACTGATGATAATGTTTATGAAAATATCACTGCAAATCTGC
lu-ecam-1     AAAATCATCTAACAAGAATAACTGATGATAATGTTTACCAAAAGATCACCGCAAAACTGC
               ** * * ** * *  ****    **  * **      * *   ** *  **   * *

pg33          CTGCAGCAGCTTCAGGAGGGACGTCCATCTGCAGCGGGCTTCGATCGGCATTTNNNACTG
ca-cc         CTCAAGAAGCTAATGGTGGAACTTCAATTTGTAGGGGGCTCAAAGCAGGATTTCAGGCAA
lu-ecam-1     CTCAAGTAGCTAATGGTGGAACTTCAATTTGTAGAGGGCTCAAAGCAGGATTCCAGGCAA
              **  ** ****   ** ** ** ** ** ** ** *****   * * * ***     *

pg33          TGATCAAGAGGAAATACCCGACGGACGGATCTGAGATTGTGTTACTGACTGACGGGGAGG
ca-cc         TTATTCAGAGTCAACAGAGCACTTCTGGTTCTGAAATCATACTACTAACCGATGGGGAAG
lu-ecam-1     TTATCCACAGTGACCAGAGTACTTCTGGTTCTGAAATCATACTATTAACTGATGGGGAAG
              * **  * **  *  *    **    ** ***** **  *  ** * ** ** ***** *

pg33          ACAACACGATAAGCGCGTGCTTCACGGAGGTGAAGCAGAGCGGAGCCATCATCCACACGG
ca-cc         ATAATGAAATACACTCATGCATTGAGGAGGTAAAACAAAGTGGTGTGATCATTCACACCA
lu-ecam-1     ATAATGAAATAAATTCATGCTTTGAGGATGTAAAACGAAGTGGTGCAATCATCCACACCA
              * **    ***    * *** *   *** ** ** *  ** ** *  ***** *****
```

```
pg33          TCGCCCTTGGGCCCTCGGCAGCGAAGGAGCTGGAGGAGCTGTCGCAGATGACAGGTGGTT
ca-cc         TTGCCCTGGGACCTTCTGCTGCCAAAGAACTGGAGACACTGTCAGATATGACAGGAGG--
lu-ecam-1     TTGCTCTGGGACCCTCTGCTGCCAAAGAACTGGAGACATTGTCAAATATGACAGGAGG--
              * ** ** ** ** ** ** ** ** ** ** ****** ****  * ******** **

pg33          TGCAGACATATGCTTCAGACCAG-GCCGAGAACAACGGCCTCATTGACGCTTTCGGGGCC
ca-cc         -----ACATCGGTTTTATGCCAATAAAGACATAAATGGCCTTACTAATGCGTTCAG----
lu-ecam-1     -----ATATCGTTTTTTTGCCAATAAAGACATAACTGGCCTTACTAATGCTTTCAG--T-
                   * **    **    ***     ** *  *  ***** * * * ** *** *

pg33          CTTTCGTCCGGGAACAGAGCTGCCTCCCAGCGCTCCATCCTTTCGGGGCCCCTTTCGTCC
ca-cc         -------------T---AGAA------------------------------TTTCATCT
lu-ecam-1     -------------A----GAA------------------------------TTTCATCT
                           *                                    **** **

pg33          GGGAACAGAGCTGCCTCCCAGCGCTCCATCCAGCTGGAGA-CAGGGCTTAACCCTCCAAG
ca-cc         AGAAGTGGCAGTATCACTCAGCAGACTATTCAGTTGGAAAGCAAAGCCTTGGCAATTACA
lu-ecam-1     AGAAGTGGAAGCATCACTCAGCAGGCTATTCAGTTGGAAAGCAAAGCCTTGAAAATTACA
               * *   *      * * ****   * ** *** **** * **  ** *        *

pg33          AAACAACGAATGGATGAATGGCACAGTGGTGGTGGACAGCACTGTTGGGGAAAGGACACT
ca-cc         GAA-AAGAAATGGGTAAATGGTACGGTGCCTGTGGATAGTACAATTGG--AAATGACACT
lu-ecam-1     GGA-AGGAAAAGAGTAAACGGCACAGTGCCTGTAGACAGTACAGTTGG--AAATGACACT
                * *   ** *  * ** ** ** ***   ** ** ** **  ****  *** ******

pg33          TTGTTTCTCATTCACCTTGGAACGGAAGTTCCTTTCCCCAATTCCTTTTTTTGGGGTTCC
ca-cc         TTCTTTGTTGT-CACATGGACAATAAAAAAGC------CAGAAATTCTTCTCCAGGATCC
lu-ecam-1     TTCTTTGTTGT-CACATGGACAATACAAAAAC------CAGAAATTGTTCTCCAAGATCC
              ** *** *  * *** * *  *    *    *      **     * ** *    * ***

pg33          CAGCGGAAGAAGCCAGGACAGTTTCTTGGTTGGAAAACACAA-CAAGATG--------GC
ca-cc         AAAAGGAAAGAAATATAAAACCTCAGATTTCAAAGAAGATAAGCTAAATATTCATTCTGC
lu-ecam-1     AAAAGGAAAGAAATATAAAACCTCGGATTTCAAAGAAGATAAGTTAAATATTCGATCTGC
               *  **** *  *  * * *  *     *  * ** * **   * **         **

pg33          TTACTTCCAAGTCCCAGGCACGGCCAAGGTTGGCATGTGGAAGTACAGCCTGNNNNNNNN
ca-cc         TCGTCTTCGAATACCTGGTATTGCAGAGACAGGTACTTGGACTTACAGCCTTCTAAATAA
lu-ecam-1     TCGTCTGCAAATACCTGGTATTGCAGAGACAGGTACTTGGACTTACAGCCTTCTAAATAA
              *    * * * * ** ** *  **  **   ** *  **** ********

pg33          NCAAGCAAGTTCACAAACCTCACTTTNGACCGTCAGCTCCCGTCGGTCCAGCGCTACCCT
ca-cc         TCATGCCAGTCCTCAAATACTAACAGTGACAGTGACCACTCGAGCAAGAAGTCCTACTAC
lu-ecam-1     TCATGCCAGCTCTCAAATGCTAACAGTGACAGTGACCACTCGAGCAAGAAGTCCTACTAT
               ** ** **  * ****    *     *** ** * * * **       **  ****

pg33          GCCTCCGGTTACCGTGACCTCTAAAATGAACAAAGACACGGGCAAATTCCCCAGCCCCAT
ca-cc         ACCCCCAGTAACTGCAACAGCTCACATGAGTCAAAATACAGCACACTACCCTAGCCCAGT
lu-ecam-1     ACCCCCAGTAATTGCAACAGCTCACATGAGTCAACATACAGCACATTATCCTAGCCCAAT
               ** ** ** *  *  **  ** * **** ** * ** *  * *  ** ***** *
```

```
pg33          GGTAGTTTACACGAAGATTCACCAAGGAACCCTGCCCATTCTCAGGGCCAAGGTCACAGC
ca-cc         GATTGTTTATGCACAAGTCAGTCAAGGGTTTTTGCCTGTACTGGGAATCAATGTAACTGC
lu-ecam-1     GATTGTTTATGCACAAGTCAGTCAAGGGTTTTTGCCTGTACTGGGAATCAGTGTAATAGC
              * * *****  *  *  *    *****     ****  * **  *   **  ** *  **

pg33          CCTGATAGAATCAGAGAATGGGAAAACAGTTACCTTGGAATTACTGGACAACGGAGCAGG
ca-cc         CATTATAGAAACTGAAGATGGCCATCAAGTAACACTGGAGCTCTGGGACAATGGTGCAGG
lu-ecam-1     CATTATAGAAACCGAAGATGGACATCAAGTAACATTGGAGCTCTGGGACAATGGTGCAGG
              * * ****** * **  ****  *   *** **  ****  *   ****** ** *****

pg33          CGCTGATGCTACCAAGAATGACGGTATCTACTCGAGGTATTTTACAGCTTACGATGCAAA
ca-cc         TGCTGATACTGTCAAGAATGATGGCATCTATTCAAGATATTTTACAGATTACCGTGGAAA
lu-ecam-1     TCGTGATACTGTCAAGAATGATGGCATCTACTCAAGATACTTTACAGATTACTATGGAAA
               **** **  ********* ** ***** ** ** ** ******* ****  ** ***

pg33          TGGTCGATACAGCGTAAAAGTGTGGGCTCTGGGAGGAGTGAACACG-CCACGCAGAAGGG
ca-cc         TGGTAGATACAGTTTAAAAGTACATGCCGAGGCAAGAAACAACACGGCTAGGCTAAGTTT
lu-ecam-1     TGGTAGATACAGTTTAAAAGTACATGCACAGGCAAGAAACAACACGGCTAGGCTAAATTT
              **** *******  *******    **   ** * **   ****** * * **  *

pg33          CACCCCCCCTCGGAGTGGAGCCATGTACATACGTGGCTGGATTGAGAATGGGGAGATCAA
ca-cc         AAGACAACCACAGAACAAAGCCCTGTATATACCAGGCTATATTGAAAATGGTAAAATTAT
lu-ecam-1     AAGACAACCACAGAACAAAGTTCTATATGTTCCAGGCTACGTTGAAAACGGTAAAATTAT
               *  *  ** * **    **   * **  * *  ****   **** ** **  * ** *

pg33          ATGGAACCCGCCGAGACCCGACATTAACAAGGACGACCTTCAGGGCAAGCAAGTGTGTTT
ca-cc         ACTGAACCCACCCAGACCTGAAGTCAAAGATGACCTGGCAAAAGCTGAAATAGAAGACTT
lu-ecam-1     ACTGAACCCACCCAGACCTGAAGTCAAAGATGACCTGGCAAAAGCTAAAATAGAAGACTT
              *  ****** ** ***** **  * **  * ***       * *   *   **     **

pg33          CAGCCGAACAGCCTCGGGGGGTTCATTCGTGGCCTCCGACGTTCCGAAGTCTCCATACCC
ca-cc         TAGCAGACTAACCTCTGGAGGGTCATTTACTGTATCAGGAGCTCC----TCCTGGTAATC
lu-ecam-1     TAGCAGACTAACCTCTGGAGGGTCATTTACTGTATCAGGAGCTCCTCC-TCCTGGTAATC
               *** **  * **** ** ** *****    *  ** *  * ***    **    **  *

pg33          GATCTTTT------CCACCCTGTAAGATCACCGACCTGAAGGCCGGGATCCAAGGGGACA
ca-cc         ACCCTTCTGTGCTCCCACCCAATAAAATTATAGACCTTGAAGCAAAATTTAAAGAAGATC
lu-ecam-1     ACCCTTCTGTGTTCCCACCCAGTAAAATTACAGATCTTGAGGCTAAGTTCAAAGAAGATT
               *** *      ******  *** ** *  ** **  * **     *  ***  **

pg33          ACCTCATCAACCTGACGTGGACGGCTCCTGGGGATGATTACGACCACGGAAGAGCTGACA
ca-cc         ACAT--TCAACTTT-CATGGACAGCCCCTGCGAATGTCCTAGATAAAGGAAAAGCCAACA
lu-ecam-1     ATAT--TCAACTTT-CATGGACAGCCCCTGGCAATGTCCTAGATAAAGGAAAAGCCAACA
              *  *  ***** *  * ***** ** **** ***     **  * **** ***  ***

pg33          GGTACATCATCAGAATAAGCACAAATATTCTTGATCTCAGAGACAAGTTCAATGATTCTG
ca-cc         GCTACATTATAAGAATAAGTAAGAGTTTCCTGGATCTCCAAAAAGATTTTGACAATGCTA
lu-ecam-1     GCTACATTATAAGAATAAGTAAGAGTTTCATGGATCGTCAAGAAGATTTTGACAATGCGA
              * ***** ** ******** *  * * *  * ****    * *  * **  *  ** *
```

```
pg33          TTCAAGTGAACACCACGGATCTCATCCCAAAGGAGGCCAACTCAGAGGAGGTCTTTGTGT
ca-cc         CTTTAGTGAATACTTCCAGTCTAAAACCTAAGGAGGCCGGCTCAGATGAAAATTTTGAAT
lu-ecam-1     CTTTAGTGAATACTTCTAATCTAATACCTAAGGAGGCCGGATCAAAAGAAAATTTTGAAT
               *  ****** **  *   *** *  ** *********   *** * **    ****  *

pg33          TTAAACCAGAAGGCATCCCTTTTACAAATGGCACGGATCTCTTCATTGCTGTACAGGCTG
ca-cc         TTAAACCAGAACCTTTTAGAATAGAAAATGGCACCAACTTCTATATTGCAGTCCAAGCCA
lu-ecam-1     TTAAGCCAGAACATTTTAGAGTAGAAAATGGCACCAAATTCTATATTTCAGTCCAAGCCA
              **** ******    *     *   *********  *  ***  *** * ** ** **

pg33          TCGATAAGACCAACCTGAAGTCAGAAATCTCCAACATTGCACAAGTATC----TTTGTTC
ca-cc         TCAATGAAGCCAATCTCACCTCAGAGGTTTCTAACATCGCACAAGCAATCAAGTTTATTC
lu-ecam-1     TCAACGAAGCCAATCTCATCTCAGAGGTTTCTCACATTGTACAAGCAATCAAATTTATTC
              ** *  *  **** ** *  *****  * **  **** * ***** *      *** ***

pg33          CTTCCCCGGAGGCTCCTCCGGAGACCTTCCGGAGACACCTGCTCCTTCTCTGCCTTGTCC
ca-cc         CTATGCCAGAAGA----C-AGTGTCCCTGCTCTGGGTACCAAGATTTCTGCAATCAATTT
lu-ecam-1     CTCTACCAGAAGA-----CAGTGTCCATGATCTGGGTACCAAGATTTCTGAAATCACTCT
              **   ** ** *        * * ** *     *    *      ****        *

pg33          TGAAATTCAGGTCAACAGCACCATTCCTGGCATTCACATTTTAAAAATTATGTGGAAGTG
ca-cc         GGCAATT----TTTGCATTAGCTATGATTTTATCTATAGTTTAAACTAGGGATTGCATCA
lu-ecam-1     GGCAATT----TTAGGATTACCAATGATTTTCTCTGTATTTTAAACTAGGAATTGTGTCA
               * ****    *    *  * *  *  *    *    * ******       * *

pg33          GCTGGGAGAATTACAGCTATCGATAGCCTAGGGCTGAGTTTTCCTGAGATAAACAAATCA
ca-cc         GAACTGAGATTCAATGTTATACATAGTT---GGCAAACATTTATTTAG--GATTTAATTT
lu-ecam-1     GCACTGATAACCAATGTTATACATAGTT---GGTACACATTTATTTAG--GATTTAATTC
              *    ** *   *  * ***  ****     **   *  ***  * **   *   ***

pg33          TCCATCCTGCTTTTGATTA-----------------------------------------
ca-cc         ACTATAC-ATTGTCTATTA-----------------------------------------
lu-ecam-1     GCTATTTTCTTGTTCTTCAGTAGCTAAATTGTGTCCAACCTTGCGACTGCAGGACTGCAG
               * **     * *   * *

pg33          --------------------------------TGAAAATTTATAAAATGCATTTT-----
ca-cc         --------------------------------TAAAGCTCTTTGAAATATATGT------
lu-ecam-1     CATGCCAGGTTTCCCTGTCCATCACCAACTCCCAGAGCTTGCTCAAATCCATGTTCATTT
                                                 *  *   * ****  ** *

pg33          ------------------------------------------------------------
ca-cc         ------------------------------------------------------------
lu-ecam-1     GAGTCAGTAATGCTAACTATCTCATCCTCTACTGCCCTCTTCTCTGTTTACCTTCAATCT


pg33          --------------AGACTTTCTGCACGAGGCAATTTA-------------ATGAAATAC
ca-cc         --------------GAACATATGAATGTTGTAAATTT-------------CCTAAATAC
lu-ecam-1     TTCCCCAGCATTAGGATCTTTTCCAATGAGTCAGCTCTTAGCATCGGGTGGCCAAAATAT
                             *    * *    *  *                   *****
```

```
pg33          ---------------AATGCT-------AAACAACTAGA--------TATGTACGTATA
ca-cc         TTGAGTTT-----ATTAGTTCT-------AATTAGTTTCA--------CTTTAAAGCAAA
lu-ecam-1     TGGCATTTTCAGCAACAGTTCTTCAAATGAAATATCCAGGGTGATTTTCTTTAGGATAGA
                          * * **       **  *                 *      * *

pg33          AAAGCTAT-------------------------TCATGTCAA---------------TAA
ca-cc         ATGAATATA-----------------------CCATTTCCTATC-----------TTAG
lu-ecam-1     CTGGTGACTGACAGTTCAAGGGACACTCTGGAGTCTTCTCCAGCACCGCACCGCAGTTTG
                    *                            * * **                 *

pg33          ATAGAAGTATGTTTT-----------------------------------------AA
ca-cc         AAAAATCCATTTATT-----------------------------------------AA
lu-ecam-1     AAAGAACCAGTTCTTTGGTACTCAGCCTTCTTTATAGTCCAATGCTCACATCTATCATGA
              * * *   *  * **                                          *

pg33          TTCAAAAAAGTGCTA---------AAAGCGGC--------------
ca-cc         CTAACCATAAAATAA---------AATGCATATTTTTA--------
lu-ecam-1     CTCCTGGAAAAACCATAGCTTTGAGAAATGGATCTTTGTTGGGAAA
               *      *     *          *
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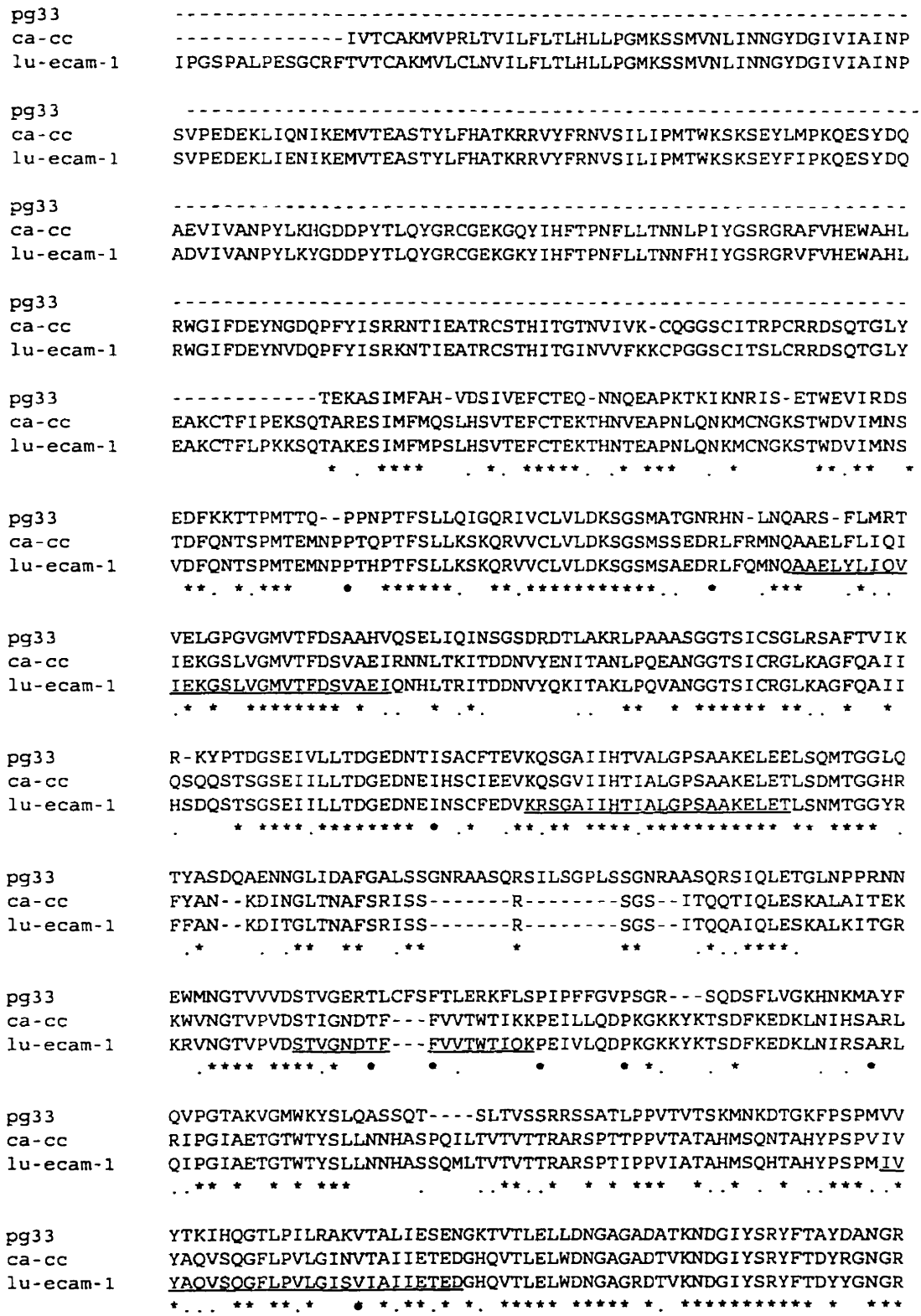
Figure 3.9. Alignment of predicted amino acid sequences for PG33, Ca-CC, and Lu-ECAM-1. Asterisks indicate amino acids that are identical in all three protein products; dots indicate conservative amino acid substitutions. Potential transmembrane domains are underlined.


pg33 ----------
ca-cc ----------IVTCAKMVPRLTVILFLTLHLLPGMKSSMVNLINNGYDGIVIAINP
lu-ecam-1 IPGSPALPESGCRFTVTCAKMVLCLNVILFLTLHLLPGMKSSMVNLINNGYDGIVIAINP
pg33 ----------
ca-cc SVPEDEKLIQNIKEMVTEASTYLFHATKRRVYFRNVSILIPMTWKSKSEYLMPKQESYDQ
lu-ecam-1 SVPEDEKLIENIKEMVTEASTYLFHATKRRVYFRNVSILIPMTWKSKSEYFIPKQESYDQ
pg33 ----------
ca-cc AEVIVANPYLKHGDDPYTLQYGRCGEKGQYIHFTPNFLLTNNLPIYGSRGRAFVHEWAHL
lu-ecam-1 ADVIVANPYLKYGDDPYTLQYGRCGEKGKYIHFTPNFLLTNNFHIYGSRGRVFVHEWAHL
pg33 ----------
ca-cc RWGIFDEYNGDQPFYISRRNTIEATRCSTHITGTNVIVK-CQGGSCITRPCRRDSQTGLY
lu-ecam-1 RWGIFDEYNVDQPFYISRKNTIEATRCSTHITGINVVFKKCPGGSCITSLCRRDSQTGLY
pg33 ----------TEKASIMFAH-VDSIVEFCTEQ-NNQEAPKTKIKNRIS-ETWEVIRDS
ca-cc EAKCTFIPEKSQTARESIMFMQSLHSVTEFCTEKTHNVEAPNLQNKMCNGKSTWDVIMNS
lu-ecam-1 EAKCTFLPKKSQTAKESIMFMPSLHSVTEFCTEKTHNTEAPNLQNKMCNGKSTWDVIMNS
pg33 EDFKKTTPMTTQ--PPNPTFSLLQIGQRIVCLVLDKSGSMATGNRHN-LNQARS-FLMRT
ca-cc TDFQNTSPMTEMNPPTQPTFSLLKSKQRVVCLVLDKSGSMSSEDRLFRMNQAAELFLIQI
lu-ecam-1 VDFQNTSPMTEMNPPTHPTFSLLKSKQRVVCLVLDKSGSMSAEDRLFQMNQAAELYLIQV
pg33 VELGPGVGMVTFDSAAHVQSELIQINSGSDRDTLAKRLPAAASGGTSICSGLRSAFTVIK
ca-cc IEKGSLVGMVTFDSVAEIRNNLTKITDDNVYENITANLPQEANGGTSICRGLKAGFQAII
lu-ecam-1 IEKGSLVGMVTFDSVAEIQNHLTRITDDNVYQKITAKLPQVANGGTSICRGLKAGFQAII
pg33 R-KYPTDGSEIVLLTDGEDNTISACFTEVKQSGAIIHTVALGPSAAKELEELSQMTGGLQ
ca-cc QSQQSTSGSEIILLTDGEDNEIHSCIEEVKQSGVIIHTIALGPSAAKELETLSDMTGGHR
lu-ecam-1 HSDQSTSGSEIILLTDGEDNEINSCFEDVKRSGAIIHTIALGPSAAKELETLSNMTGGYR
pg33 TYASDQAENNGLIDAFGALSSGNRAASQRSILSGPLSSGNRAASQRSIQLETGLNPPRNN
ca-cc FYAN--KDINGLTNAFSRISS-------R--------SGS--ITQQTIQLESKALAITEK
lu-ecam-1 FFAN--KDITGLTNAFSRISS-------R--------SGS--ITQQAIQLESKALKITGR
pg33 EWMNGTVVVDSTVGERTLCFSFTLERKFLSPIPFFGVPSGR---SQDSFLVGKHNKMAYF
ca-cc KWVNGTVPVDSTIGNDTF---FVVTWTIKKPEILLQDPKGKKYKTSDFKEDKLNIHSARL
lu-ecam-1 KRVNGTVPVDSTVGNDTF---FVVTWTIQKPEIVLQDPKGKKYKTSDFKEDKLNIRSARL
pg33 QVPGTAKVGMWKYSLQASSQT----SLTVSSRRSSATLPPVTVTSKMNKDTGKFPSPMVV
ca-cc RIPGIAETGTWTYSLLNNHASPQILTVTVTTRARSPTTPPVTATAHMSQNTAHYPSPVIV
lu-ecam-1 QIPGIAETGTWTYSLLNNHASSQMLTVTVTTRARSPTIPPVIATAHMSQHTAHYPSPMIV
pg33 YTKIHQGTLPILRAKVTALIESENGKTVTLELLDNGAGADATKNDGIYSRYFTAYDANGR
ca-cc YAQVSQGFLPVLGINVTAIIETEDGHQVTLELWDNGAGADTVKNDGIYSRYFTDYRGNGR
lu-ecam-1 YAQVSQGFLPVLGISVIAIIETEDGHQVTLELWDNGAGRDTVKNDGIYSRYFTDYYGNGR

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pg33          YSVKVWALGGVNTPRR--RAPP---LGVEPCTYVAGLRMGRSNGTRRDPTLTRTTFRASK
ca-cc         YSLKVHAEARNNTARLSLRQPQNKALYIPGYIENGKIILNPPRPEVKDDLAKAEIEDFSR
lu-ecam-1     YSLKVHAQARNNTARLNLRQPQNKVLYVPGYVENGKIILNPPRPEVKDDLAKAKIEDFSR
              **.** *    ** *   * *    * .         . ..      .*          *.

pg33          CVSAEQPRGVHSWPPTFRSLHTRSFPPCKITDLKAGIQGDNLINLTWTAPGDDYDHGRAD
ca-cc         LTSGGS-FTVSGAPP---GNHPSVLPPNKIIDLEAKFKED-HIQLSWTAPANVLDKGKAN
lu-ecam-1     LTSGGS-FTVSGAPPP--GNHPSVFPPSKITDLEAKFKED-YIQLSWTAPGNVLDKGKAN
                *      *   **     *    ** ** ** *  . *  *.*.****    *.*.*

pg33          RYIIRISTNILDLRDKFNDSVQVNTTDLIPKEANSEEVFVFKPEGIPFTNGTDLFIAVQA
ca-cc         SYIIRISKSFLDLQKDFDNATLVNTSSLKPKEAGSDENFEFKPEPFRIENGTNFYIAVQA
lu-ecam-1     SYIIRISKSFMDRQEDFDNATLVNTSNLIPKEAGSKENFEFKPEHFRVENGTKFYISVQA
               ******   .* .  *  .  ***. * ****.* * * ****     ***  .*.***

pg33          VDKTNLKSEISNIAQVSLFLPR------RLLRRPSGDTCSFSALS------
ca-cc         INEANLTSEVSNIAQAIKFIPMPEDSVPALGTKISAINLAIFALAMILSIV
lu-ecam-1     INEANLISEVSHIVQAIKFIPLPEDSVHDLGTKISEITLAILGLPMIFSVF
              .  .** **.*.* *   *.*       *  . *  .  .   *
```

products was subcloned into EcoRV-cut pDK101, and is referred to as PG33e. PG33e is 683 base pairs in size (Figure 3.4A, lane 3), and strongly cross-reacts in a Southern blot with a probe constructed from internal sequences from the porcine clone PG33d (Figure 3.4B).

The 5' end of the PG33d clone has a 188-base overlap with the 3' end of the PG33e clone, which allows a comparison to be made between the nucleotide and predicted amino acid sequences of the porcine and human PG33 genes. Nucleotide homology between the two genes in this region is 80% (Figure 3.6A). The predicted amino acid sequence has an 89% identity, which increases to 95% if conservative amino acid substitutions are included in the calculation (Figure 3.6B).

The full PG33 sequence obtained to date includes a 2322-base pair cDNA of which a 2040-base open reading frame encodes a 679-amino acid protein product (Figure 3.7). Hydrophobicity analysis of the predicted amino acid sequence indicates four potential transmembrane domains. Examination of the predicted amino acid sequence reveals the presence of 3 potential phosphorylation sites for protein kinase A and cGMP-dependent protein kinase, 15 potential phosphorylation sites for protein kinase C, 7 potential phosphorylation sites for CaMK II, and 8 potential sites for phosphorylation by casein kinase II. The predicted protein product also contains 6 consensus sites for N-linked glycosylation.

The cDNA sequence of PG33, when originally compared to known sequences, matched no DNA sequences contained in the GenBank database. Two recently identified genes, however, have been found to contain significant nucleotide and amino acid homology to the PG33 cDNA and predicted protein product. The first gene was identified in bovine tracheal epithelium and encodes the calcium-regulated chloride channel Ca-CC (Cunningham *et al.*, 1995). The PG33 cDNA cloned so far has a 60% nucleotide homology to the bovine chloride channel (Figure 3.8). Based on the deduced amino acid sequence, there is a 43% amino acid identity, increasing to 57% if conservative substitutions are also included (Figure 9).

The second gene with significant homology to the PG33 cDNA is lung-

endothelial cell adhesion molecule-1 (Lu-ECAM-1), isolated from bovine aortic endothelial cells and thought to be involved in the organ-specific metastasis of tumor cells (Elble *et al.*, 1997). Nucleotide homology between the two cDNAs is 61% (Figure 3.8), while amino acid identity is 43%; the DNA sequence homology observed between Ca-CC and Lu-ECAM-1 is 92%, while amino acid homology between the two is 88% (Elble *et al.*, 1997).

3.4 Discussion

The technical difficulties associated with the purification of integral membrane proteins such as ion channels are considerable, as evidenced by the limited success of previous attempts by this laboratory to isolate the protein species responsible for the chloride conductive pathway under investigation. Molecular biological techniques offer an alternative approach to the study of protein function, in which the isolation of the gene for the protein of interest provides a potential source from which a purified protein product can be translated *in vitro*.

Use of an anti-chloride conductance monoclonal antibody to screen a porcine expression library resulted in the identification of the apparently novel, though not full-length, cDNA clone PG33. Based on Northern analysis of poly A+ RNA purified from porcine parotid salivary gland, the estimated size of the PG33 transcript is 2.7 kb. Repeated attempts to obtain a transcript size from Northern blots of porcine small intestinal RNA were unsuccessful. This does not necessarily indicate that PG33 mRNA is not expressed in the small intestine, since this message can be successfully amplified from ileal RNA by reverse transcriptase PCR (see Chapter 4: Tissue Expression).

Two factors appear to contribute to the negative results of the small intestinal Northerns. The first is the low copy number of the PG33 message; based on the amount of template required to amplify PG33 by RT-PCR, the copy number is 0.01% or less. Furthermore, extremely large amounts of polyA+ RNA (20 μg) were required to visualise the PG33 transcript in the Northern blot of parotid RNA. Second, exposure to extracellular RNases known to be present in the intestinal lumen may contribute to the

deterioration of the sample during RNA purificiation from small intestinal tissue, exacerbating the experimental difficulties.

Cloning of PG33 cDNA through a variety of approaches has resulted in a current total length of 2322 base pairs of confirmed sequence. The open reading frame is continuous throughout the five PG33 clones, of which four were isolated from the porcine cDNA library and one from a human intestinal library. Nucleotide and amino acid homology between the pig and human sequences is extremely high. The conservation of amino acid sequence between the two species suggests that the translated protein product of a porcine-human chimera would not have a significantly different three-dimensional structure than a protein translated from an entirely porcine cDNA.

Comparison of the PG33 cDNA sequence with sequences from DNA and protein databases reveals significant homology with only two reported sequences. The protein product of one of these genes, Ca-CC, has been shown to function as a calcium-regulated chloride channel in bovine tracheal epithelium (Cunningham *et al.*, 1995). This is consistent with the hypothesized role of the related PG33 gene product in intestinal epithelium.

The other gene, however, encodes an endothelial cell adhesion molecule (Lu-ECAM-1) that appears to promote tumor metastasis to lung tissue (Elble & Pauli, 1996; Elble *et al.*, 1997). There are other instances in which ion channel activity and cell adhesion function have been reported in a single protein; for example, the *Caenorhabditis elegans unc-105* gene product is a homolog of the degenerin/ENaC superfamily of sodium channels, and this gene product is thought to function in mechanosensation through an interaction with type IV collagen (Liu *et al.*, 1996). The Lu-ECAM-1 protein has not yet been shown to function as a chloride channel, though considering its extremely high (88%) amino acid homology with the Ca-CC gene product, it would perhaps be surprising if Lu-ECAM-1 did not exhibit channel activity.

An examination of the deduced amino acid sequence of the PG33 protein product reveals the presence of four regions of relatively high hydrophobicity which

might potentially act as transmembrane domains. These regions, though predominantly hydrophobic, also contain basic residues, which is consistent with a possible role as a membrane channel through which negatively charged chloride ions may pass. Comparison of the amino acid sequences of PG33, Ca-CC, and Lu-ECAM-1 demonstrates that three of the four hydrophobic regions are well conserved between the three proteins (Figure 9).

The protein sequence of the PG33 gene product also contains six consensus sites for N-linked glycosylation. This is consistent with the apparent glycosylation status of the antigen against which the anti-chloride conductive antibody was raised. Since the antigen could only be immunoprecipitated from a complex population of brush-border membrane proteins when tunicamycin pretreatment prevented protein glycosylation (Racette *et al.*, 1996), it seems likely that the antigen was itself a target for N-linked glycosylation.

Further analysis of the amino acid sequence identified a number of potential phosphoacceptor sites for phosphorylation by multiple serine/threonine protein kinases. The majority of the potential phosphorylation sites are for protein kinase C and Ca^{2+}/calmodulin kinase II, though there are also several for casein kinase II. Only a few potential sites for phosphorylation by PKA or PKG occur in the PG33 protein sequence. Which, if any, of these sites act as phosphoacceptors *in vitro* or *in vivo* has yet to be determined. All of the sites identified appear to be relatively "strong", or classical, consensus sequences for phosphorylation by each protein kinase.

While the precise locations of the consensus sequences in PG33 differ from those in Ca-CC, the overall pattern is similar: multiple sites for PKC, CaMK II, and casein kinase II, but few for PKA or PKG (Cunningham *et al.*, 1995). Ca-CC also contains consensus sites for phosphorylation by tyrosine kinase which are not found in PG33 (Cunningham *et al.*, 1995). Under physiological conditions, the primary control over Ca-CC activity appears to be through the activity of CaMK II (Fuller *et al.*, 1994). If the PG33 cDNA encodes a similar chloride channel, potential regulatory mechanisms will require investigation.

One area in which PG33 differs from Ca-CC and Lu-ECAM-1 is the relative sizes of the cDNAs. The full-length bovine cDNA is reported to be 3001 bases, however, with a 2712-base continuous open reading frame encoding a 903-amino acid protein product (Cunningham *et al.*, 1995). Northern analysis of bovine tracheal mRNA shows a single band at 3.1 kb. This is somewhat larger than the estimated size of the PG33 transcript, which is 2.7 kb including untranslated regions. The cDNA sequence of Lu-ECAM-1 was obtained from a number of overlapping clones. The first, a 3.3 kb sequence encoding a 905-amino acid protein product, was isolated by PCR from reverse transcribed bovine aortic endothelial cell (BAEC) RNA. The other three clones, of the same size and smaller (3.3, 2.8, and 1.3 kb) were isolated from a BAEC cDNA library. Northern analysis of BAEC RNA showed the presence of multiple bands, consistent with these sizes, and the major Lu-ECAM-1 protein species observed in this tissue are thought to be the result of proteolytic processing of a single initial translational product (Elble *et al.*, 1997).

The Ca-CC and Lu-ECAM-1 cDNAs align with only very minor gaps between the coding portions of the two sequences, consistent with the high degree of homology shared by these two genes. The PG33 cDNA sequence also aligns well with Ca-CC and Lu-ECAM-1 for the majority of the sequence. There is one region, however, in which the nucleotide and amino acid sequence of PG33 diverges from that of Ca-CC and Lu-ECAM-1. The 17-amino acid sequence between Ser-249 and Ser-265 of the PG33 protein product does not have a counterpart in the sequence of either the Ca-CC or the Lu-ECAM-1 protein. Furthermore, the first 12 residues of the region beginning at S249 (SSGNRAASQRSI) are repeated following the serine residue at position 265. The remaining 5 residues are not repeated. This repeated sequence may be the result of an exon duplication that did not occur in either Ca-CC or Lu-ECAM-1.

In summary, the isolation of the PG33 cDNA has identified the third member of a novel gene family. One of the other members of this family, Ca-CC, has been shown to act as a calcium-regulated chloride channel. The other, Lu-ECAM-1, is known to function in cell adhesion, but as its discovery is very recent, no reports have yet been

published concerning a possible role as a chloride channel. The use of an anti-chloride conductance antibody to select PG33 as well as its sequence homology to Ca-CC and Lu-ECAM-1 are consistent with a role for this gene product in ion transport.

Testing of this hypothesis will require the isolation of the remainder of the PG33 sequence and the construction of a full-length chimera. The protein product of this chimeric cDNA can then be expressed, reconstituted into a planar lipid bilayer system, and definitively tested for chloride channel function.

4.0 Expression of PG33 in Porcine Tissues

4.1 Introduction

The presence of channel proteins in cell membranes permits the passage of ions across the otherwise relatively impermeable lipid membrane; the movement of these ions is involved in a number of critical processes in both epithelial and nonepithelial cell types. Linked to the osmotic movement of water, electrolyte transport controls net fluid absorption and secretion across epithelial barriers. Ion transport also plays important roles in the regulation of cell volume and internal pH, as well as in maintaining the cell membrane potential.

Multiple channel types are involved in the transport of each of the major electrolytes. Some of these channels are found in only a single cell type, while others are broadly distributed. Presumably, the tissue distribution of a channel type is related to its physiological function: widely expressed channels may be involved in essential processes common to many different cell types, while tissue-specific channels may play roles in the functions of specialised cells.

Widely expressed chloride channels include some members of the ClC family, the cystic fibrosis transmembrane conductance regulator (CFTR), and the outwardly rectifying chloride channel (ORCC), while other members of the ClC family have a restricted tissue expression.

The ClC family contains a number of structurally related genes, many of which have been shown to act as voltage-gated chloride channels. The first member of the family, ClC-0, was originally cloned from the electric organ of *Torpedo marmorata*, and is involved in the generation of electric current by this organ (Jentsch *et al.*, 1990). In rapid succession thereafter, several homologous genes were cloned and their

skeletal muscle tissue and is defective in the disorder myotonia (Steinmeyer *et al.*, 1991), though ClC-1 mRNA can be detected in lower levels in other tissues. The ClC-K subfamily, including ClC-K1 and ClC-K2, is expressed in the highest levels in the kidney (Uchida *et al.*, 1993), and may be involved in the regulation of urine concentration (Uchida *et al.*, 1993; Pusch & Jentsch, 1994). Another recently cloned family member, ClC-5, is also kidney-specific (Lloyd *et al.*, 1996; Sakamoto *et al.*, 1996).

Several members of the ClC family have a broader tissue expression than ClC-1 and the ClC-K subfamily. ClC-2 has a nearly ubiquitous pattern of tissue distribution; its mRNA is detectable not only in epithelial tissues such as pancreas, liver, intestine, stomach, and the cell lines CFPAC-1 and T84, but also in nonepithelial tissues including heart, brain, 3T3 fibroblasts, and the Chinese hamster ovary (CHO) cell line (Thiemann *et al.*, 1992). Expressed in *Xenopus* oocytes, ClC-2 acts as a chloride channel that can be activated by strong hyperpolarizing voltages (Thiemann *et al.*, 1992) and by exposure to hypotonic solutions (Grunder *et al.*, 1992). Because the voltages required to activate ClC-2 are nonphysiological, however, it has been suggested that the more likely physiological activator of ClC-2 function is altered cell volume (Grunder *et al.*, 1992; Pusch & Jentsch, 1994).

ClC-6 and ClC-7 are widely expressed in both rat and human tissues, including brain, testis, ovary, skeletal muscle, small and large intestine, pancreas, and lung (Brandt & Jentsch, 1995). These gene products are currently classified as putative chloride channels, since neither has yet been shown to cause chloride channel activity in *Xenopus* expression systems (Brandt & Jentsch, 1995; Buyse *et al.*, 1997).

Other chloride channels with a wide tissue distribution include CFTR as well as the outwardly rectifying chloride channel (ORCC). It seems clear, based on the severe and widespread clinical pathology that results from defective CFTR expression, that this chloride channel plays a major role in the control of fluid and electrolyte secretion in many exocrine tissues. While tissue expression of CFTR correlates in general with the characteristic histopathology of the disease, not all tissues that express CFTR exhibit

characteristic histopathology of the disease, not all tissues that express CFTR exhibit morphological changes as a result of chloride impermeability through this channel.

CFTR is strongly expressed throughout the small intestine, with a decreasing gradient of expression both from crypt to villus and from duodenum to ileum (Tresize & Buchwald, 1991; Strong *et al.*, 1994). CFTR message can also be detected in pancreatic intercalated ducts and interlobular ducts (Tresize & Buchwald, 1991; Strong *et al.*, 1994) and in the epithelial lining of the gallbladder and colon (Strong *et al.*, 1994).

Moderate levels of CFTR expression can be detected in airway epithelia, with higher levels in the underlying submucosal glands (Engelhardt *et al.*, 1992). Other tissues in which CFTR is expressed include the intercalated and interlobular ducts of the major salivary glands, the secretory coils of apocrine sweat glands, thyroid follicular cells, epididymal cells in the testis, and the uterine endometrium (Tresize & Buchwald, 1991; Crawford *et al.*, 1991; Tresize *et al.*, 1992; Tresize *et al.*, 1993; Devuyst *et al.*, 1997). In addition to its presence in these native tissues, CFTR is also endogenous to many immortalized cell lines such as the intestinal cell lines T84, CaCo-2, and HT-29, as well as a number of cultured airway and pancreatic cell lines (Riordan *et al.*, 199, 1989; Ward *et al.*, 1991; Crawford *et al.*, 1991).

Although it is predominantly expressed in epithelial tissues, CFTR can also be found in a variety of nonepithelial tissues including the heart (Levesque *et al.*, 1992), lymphocytes (McDonald *et al.*, 1992), and the kidney (Crawford *et al.*, 1991). While overt pathology has not been described in these tissues in cystic fibrosis, cAMP-regulated currents are defective in CF lymphocytes (McDonald *et al.*, 1992), and CFTR-mediated currents have been suggested to play a role in cells affected by autosomal polycystic kidney disease (Hanaoka *et al.*, 1996).

Because the gene encoding the ORCC has not yet been cloned, cDNA probes are not available for in situ hybridization studies. Consequently, the presence of the channel in a given tissue or cell must be confirmed by functional tests such as its activation in excised membrane patches by strong depolarization. Nonetheless, the outwardly rectifying chloride channel appears to have a tissue distribution similar,

though not identical, to that of CFTR. The ORCC can be found in epithelia from the small and large intestine (Halm & Frizzell, 1992), the pancreatic ducts (Ward *et al.*, 1991), airways (Welsh, 1986), and sweat gland (Krouse *et al.*, 1989), as well as in nonepithelial tissues such as lymphocytes and the heart (Garber, 1992; Duan *et al.*, 1997).

A number of chloride channel species have been described as having a restricted tissue expression. In addition to members of the ClC family such as ClC-1, ClC-K1 and ClC-K2, a calcium-activated chloride channel (Ca-CC) purified from bovine trachea (Ran & Benos, 1991; Ran & Benos, 1992) was not detected in any of the other bovine tissues examined (Cunningham *et al.*, 1995). As well, neuronal chloride channels gated by amino acids such as GABA and glutamate are involved in transmission of nerve impulses and are restricted to the central nervous system (Cull-Candy & Ogden, 1985).

PG33 was originally identified in small intestinal epithelium, which traditionally exhibits both secretory and absorptive abilities. Though the only known homologue of PG33 is the calcium-regulated chloride channel Ca-CC, whether the PG33 gene product can also function as a chloride channel has not yet been established. It was thought important, however, to investigate the tissue distribution of PG33 in order to compare its expression to that of known chloride channels such as CFTR, the ORCC, and the bovine calcium-regulated channel. Expression of PG33 in many tissues might indicate a housekeeping function for this gene product. Alternatively, if the expression of PG33 is restricted to certain tissues, the identification of such tissues might provide information suggesting a more specialised role for the PG33 protein.

In addition, the PG33 cDNA was selected through the use of an anti-chloride conductance monoclonal antibody raised against small intestinal brush-border membrane antigens. In immunohistochemical studies, this antibody identified the crypt region as the primary location of this antigen in porcine small intestine. Presence of the PG33 message should, in theory, correlate with expression of antigen recognised by the monoclonal antibody.

The simplest and most sensitive technique which would allow both of these

questions to be investigated is reverse transcriptase PCR (RT PCR). As well, in situ hybridization studies were undertaken, both to confirm the results of the RT PCR experiments and to identify the specific cell types within porcine tissues in which the PG33 message is expressed.

4.2 Methods

4.2.1 Reverse Transcriptase PCR

Total RNA was purified according to the acid guanidium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (1987). Postmortem tissue samples were removed from weanling pigs of mixed Yorkshire-Landrace breed (through kind permission of Dr. Linda Hiebert), and homogenized in 4 M guanidium isothiocyanate, 25 mM sodium citrate pH 7, and 0.5% laurylsarcosine (solution D). The samples were then repeatedly extracted in 0.1 volumes 2 M sodium acetate (pH 4), 1 volume of phenol, and 0.2 volumes of a 24:1 chloroform-isoamyl alcohol mix until no interface was visible between the aqueous and organic phases. RNA was precipitated by the addition of 1 volume of isopropanol at -20°C for an hour followed by a 15 minute centrifugation at 9000 x g. The pellet was resuspended in solution D and extracted once again, then reprecipitated as described above. The resulting pellet was washed with 95% ethanol, air dried, then resuspended in DEPC-treated water.

The template for each reverse transcription reaction was 5 μg total RNA, denatured by heating at 90°C for 5 minutes immediately before the initiation of the reaction. Generation of cDNA from the RNA was accomplished in a reaction solution containing 50 mM Tris-HCl pH 8.15, 6 mM $MgCl_2$, 40 mM KCl, 1 mM DTT, 1 mM each dGTP, dATP, dCTP, dTTP, 33.7 units RNAGuard (Pharmacia), 100 units Moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco BRL), and 1 pmol of the 241a antisense primer [5'AATGCCAGGAATGGT]. The reaction was allowed to continue for 30 minutes at 42°C, then the enzyme was denatured in a 5-minute heat treatment at 94°C.

One μL of the reverse transcription was used as a template for amplification of

internal PG33 sequences. The PCR reaction conditions were identical to those previously described, except that the primers used to amplify the internal PG33 segment were the 241a antisense primer and the 911s sense primer [5' GGTCGATACAGCGTA], and the total number of cycles was 30.

4.2.2 In situ Hybridization

4.2.2.1 Preparation of Tissue Sections

Postmortem tissue samples were removed from experimental pigs as described above. Sections measuring approximately 10 mm x 10 mm x 3 mm were fixed for 3 hours in an ice-cold solution containing 85% ethanol, 5% glacial acetic acid, and 4% formaldehyde. Tissue sections were then stored in 70% ethanol at -20°C until processed.

Processing involved sequential one-hour exposures to formalin, 70% ethanol, 80% ethanol, and 95% ethanol, followed by 3 hours in absolute ethanol, 3 hours in xylene, and 4 hours in paraffin. Samples were then embedded in paraffin cassettes and stored at room temperature until thin-sectioned. Sequential thin sections were sliced by microtome onto 3-aminopropyltriethoxylsilane (TESPA)-treated glass slides and dried overnight at 42°C.

4.2.2.2 Probe Synthesis

Synthesis of the ^{35}S-labelled probes was carried out for 90 minutes at 37°C in reactions containing 40 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 10 mM NaCl, 2 mM spermidine, 100 mM DTT, 0.5 mM each ATP, CTP, GTP, 12.5 μM UTP, 25 pmol ^{35}S-UTP, 0.5 units Rnasin (Promega), 50 units RNA polymerase (Promega), and 2 μg linearised template. The sense probe was constructed from Apa I-cut PG33d template, and the Sp6 RNA polymerase (Promega) was used to accomplish cRNA synthesis. Antisense cRNA was produced using T7 RNA polymerase (Gibco BRL) on Kpn I-cut PG33c.

Template DNA was then removed by the addition of 1 unit RNasin, 10 units RNase-free DNase I (Promega), 20 μg yeast tRNA, and a further incubation at 37°C for 15 minutes. The unincorporated nucleotides, enzymes, and salts were removed with the RNaid RNA purification kit (BIO 101), and the labeled cRNA was eluted into a 50% formamide solution.

4.2.2.3 Pre-hybridization Treatment of Tissue Sections

Tissue sections were dewaxed and rehydrated by a 10-minute bath in xylene followed by a second, 5-minute bath in xylene. Sections were then exposed to 100%, 90%, 70%, and 30% ethanol for 30 seconds each. Hydrolysis of protein crosslinks was initiated in a 20 minute bath in 0.2 M HCl; the acid was then removed in a 5 minute rinse in TE. Further protein hydrolysis was accomplished by a 15 minute incubation at 37°C in proteinase K (20 μg/mL) in TE. Proteinase K was neutralised by a 2-minute exposure to 0.2% glycine in PBS, followed by a 3-minute equilibration in PBS. Tissue RNases were neutralised by a 20-minute post-fixing on ice in 4% paraformaldehyde in PBS, and the paraformaldehyde was then removed in a 5-minute PBS wash. Non-specific binding sites were blocked by a 10-minute exposure to 0.1 M triethanolamine in 0.5% acetic anhydride. The sections were then left to equilibrate in 2x SSC.

4.2.2.4 Hybridization and Post-Hybridization Washes

Tissue sections were hybridized overnight at 50°C in a solution containing 10 mM Tris-HCl pH 7.6, 300 mM NaCl, 5 mM EDTA, 10 mM DTT, 10% dextran sulphate, 50% formamide, 100 μg/mL yeast tRNA, 500 µM uridine 5' [α-thio] triphosphate (thio-UTP), and 0.25 ng/mL ^{35}S-labeled cRNA probe.

Coverslips were removed from the slides in 2x SSC, and the sections washed twice for 30 minutes at 50°C in 50% formamide, 2x SSC, 10 mM 2-mercaptoethanol. Sections were equilibrated at 37°C for 15 minutes in 4x SSC in TE, then the nonspecifically bound ^{35}S-cRNA was digested by a 30-minute exposure to 20 μg/mL RNase A in 4x SSC in TE at 37°C. RNase was removed by a 30-minute wash at 37°C

in 4x SSC in TE, and the digested ^{35}S-UTP was then removed by washing in 50% formamide, 2x SSC, 10 mM 2-mercaptoethanol for an hour at 50°C. The sections were equilibrated briefly at room temperature in 2x SSC, then dehydrated by 30-second exposures to 30%, 50%, 70%, and 93% ethanol, all in 0.3 M ammonium acetate. Dehydration was completed by a 30-second exposure to absolute ethanol and the sections were then allowed to air dry.

4.2.2.5 Autoradiography and Staining

Slides were dipped in photographic emulsion (Kodak), air-dried, then exposed at -20°C for 3 to 14 days. Slides were developed at room temperature for 2.5 minutes in Kodak D19 developer, washed briefly in water, and fixed for 5 minutes in Kodak rapid fixer.

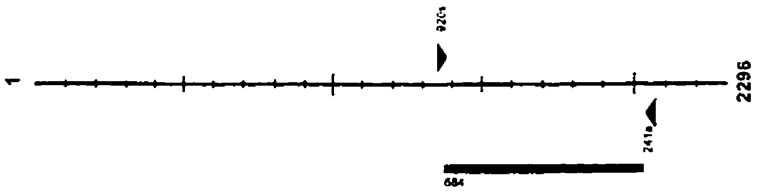
After an overnight soak in water, slides were dehydrated for 2 to 3 minutes in 60% ethanol. They were then stained for 30 seconds-3 minutes in 0.2% toluidine blue in 60% ethanol and washed briefly in water. The final stages involved two 2.5-minute washes in acetone and two 2.5-minute washes in xylene to dehydrate the stained emulsion. Coverslips were fixed in place with Permount (Fisher).

4.3 Results

PG33 message was detected in pig ileal total RNA samples using the reverse transcriptase PCR technique (Figure 4.1, lane 2). This validates the use of the anti-intestinal chloride conductance monoclonal antibody to screen an intestinal expression library, since the antibody was raised against protein from the apical membrane of intestinal brush-border epithelial cells. The antibody subsequently reacted with a fusion protein product encoded by a cDNA from the expression library, and this cDNA had been reverse transcribed from poly A+ RNA purified from porcine small intestinal epithelia.

While PG33 message was detected in small intestinal mucosa, it was not detected in total RNA from large intestinal mucosal samples (Figure 4.1, lane 3).

A.



B.

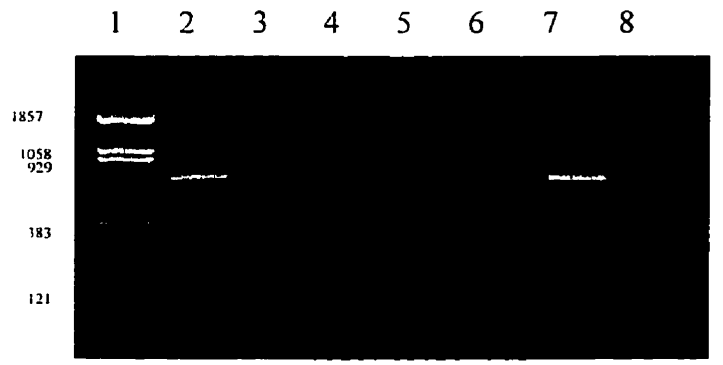


Figure 4.1. Intestinal and airway expression of PG33. Five µg total RNA was reverse transcribed with MMLV-reverse transcriptase and the 214a antisense primer, then PCR-amplified for 30 cycles using the PG33 internal primers 241a and 911s, then visualised in a 0.8% agarose gel containing 0.25 µg/mL ethidium bromide. A: Schematic of PG33 indicating the internal sequence amplified by 241a and 911s. B: RT PCR amplification of PG33. Lane 1: pBR322-BstN I standards (1857, 1058, 929, 383, and 121 bp). Lane 2: small intestine. Lane 3: colon. Lane 4: trachea. Lane 5: bronchi. Lane 6: lung. Lane 7: PG33 positive control. Lane 8: no template control.

Hence, the expression of PG33 may be restricted to specific regions within the gastrointestinal tract. Likewise, PG33 message was detected in samples from tracheal epithelia (Figure 4.1, lane 4), but not in samples taken from large bronchial epithelia (Figure 4.1, lane 5) or from lung parenchyma (Figure4. 1, lane 6).

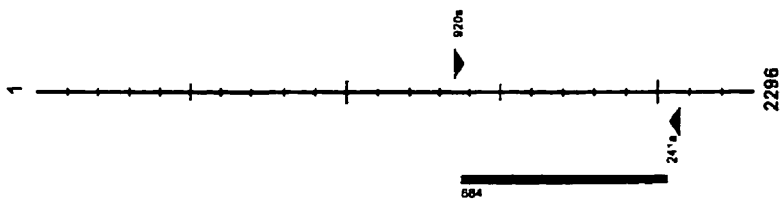
The expression of PG33 in other exocrine tissues was also investigated. Total RNA was purified from tissue samples taken from the major salivary glands of the pig, which are the parotid, mandibular, and sublingual glands. The minor salivary glands, including the buccal, labial, and pharyngeal glands were not examined. PG33 message was detected in samples from all three major salivary glands (Figure 4.2, lanes 3-5). While RT PCR is not a strictly quantitative procedure, a rough estimate of relative message abundance may be made since the amount of total RNA reverse transcribed was the same in all tissue samples. Consequently, of the major salivary glands, the expression of PG33 appears to be highest in the parotid, followed by the sublingual, and lowest in the mandibular gland.

Reverse transcriptase PCR was also performed on pancreatic total RNA samples. Surprisingly, considering the classically exocrine nature of the pancreas, no PG33 message was detected in these samples (Figure 4.2, lane 2). The expression of PG33 in a number of other tissues was also investigated. Total RNA was purified from heart, skeletal muscle, stomach, liver, and kidney, and PG33 message was not detected in any of these samples (Figure 4.3, lanes 2-6).

In summary, of all the tissues examined using the reverse transcriptase PCR technique, PG33 message was only detected in the small intestine, the trachea, and the parotid, mandibular, and sublingual salivary glands of the pig.

Although RT PCR has the advantage of being an extremely sensitive method of detection, this technique does not allow the identification of the specific cell types within a tissue that express the genes of interest. In situ hybridization, on the other hand, permits the investigation of gene expression from a histological perspective. The expression of PG33 was therefore studied by in situ hybridization in a number of porcine tissues which had been previously identified by RT PCR as either expressing or

A.



B.

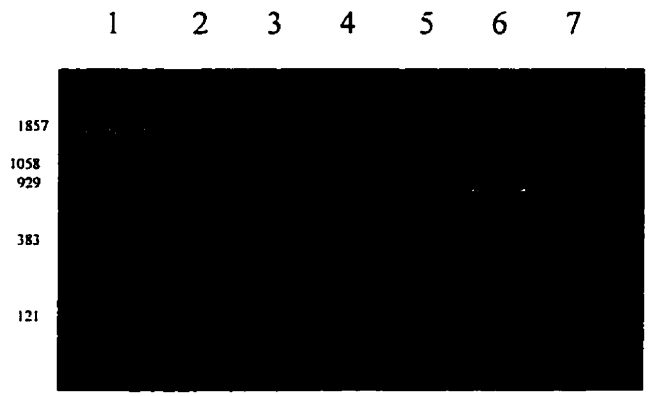
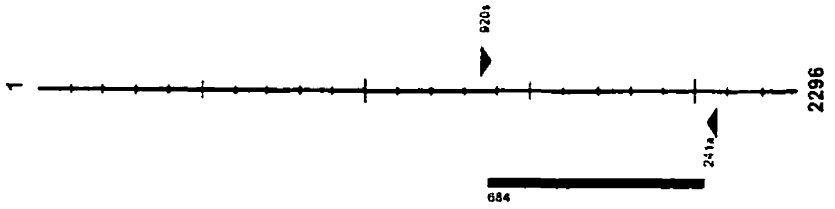


Figure 4.2. Pancreatic and salivary gland expression of PG33. Five μg total RNA was reverse transcribed with the 241a antisense primer and PCR-amplified for 30 cycles using the PG33 internal primers 241a and 911s, then visualised in a 0.8% agarose gel containing 0.33 μg/mL ethidium bromide. A: Schematic of PG33 indicating the 684-bp internal sequence amplified by 241a and 911s. B: RT PCR amplification of PG33. Lane 1: pBR322-BstN I standards (1857, 1058, 929, 383, and 121 bp). Lane 2: pancreas. Lane 3: parotid gland. Lane 4: submandibular gland. Lane 5: sublingual gland. Lane 6: PG33 positive control. Lane 7: no template control.

A.



B.

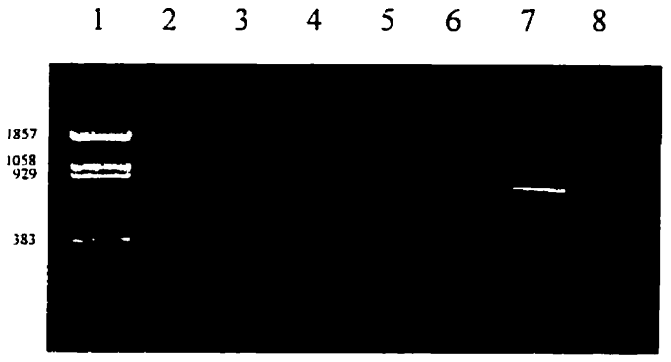


Figure 4.3. Expression of PG33 in heart, skeletal muscle, stomach, liver, and kidney. Five μg total RNA was reverse transcribed with the 241a antisense primer and PCR-amplified for 30 cycles using the PG33 internal primers 241a and 911s. Products were visualised in a 0.8% agarose gel containing 0.33μg/mL ethidium bromide. A: Schematic indicating the PG33 internal sequence amplified by 241a and 911s. B: RT PCR amplification of PG33. Lane 1: pBR322-BstN I standards (1857,1058, 929, 383, and 121 bp). Lane 2: heart. Lane 3: skeletal muscle. Lane 4: stomach. Lane 5: liver. Lane 6: kidney. Lane 7: PG33 positive control. Lane 8: no template control.

not expressing this gene. This included ileum and trachea, both of which were positive for PG33 expression in the RT PCR studies, and heart, pancreas, colon, and lung, tissues in which PG33 message had not been detected in the RT PCR studies.

The in situ hybridization experiments confirmed the expression of PG33 in the small intestine and the trachea. In the trachea, PG33 message was detected in the surface epithelium (Figure 4.4, panels B and C). The underlying submucosal glands also exhibited an extremely strong signal, but no signal was apparent elsewhere within the submucosal layer (Figure 4.4, panels A and B). Expression of PG33 message appeared to be highest in the glands themselves, rather than the epithelium lining the collecting ducts opening onto the tracheal surface. Within the submucosal glands, serous cells could not be distinguished from mucus-producing cells at the level of resolution available. The tracheal lamina propria, cartilage, and adventitia were all negative (Figure 4.4, panel A). No signal was observed in the sense control sections (Figure 4.4, panel D).

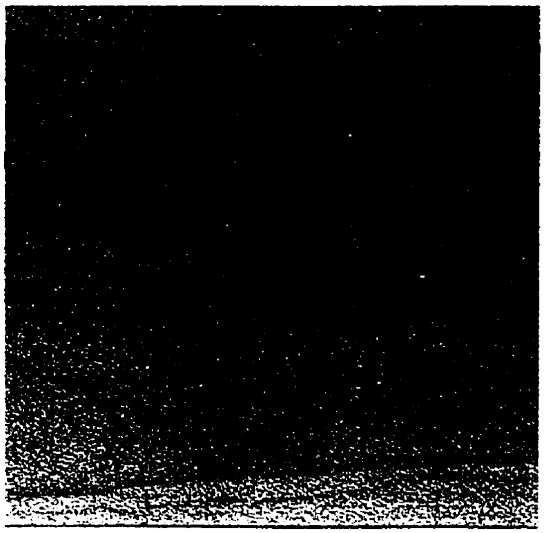
In the ileum, the epithelial cells in both the crypts and villi exhibited a diffuse but moderately strong signal, indicative of PG33 expression throughout this region (Figure 4.5, panel B; Figure 4.6, panel B). No gradient of distribution was apparent along the crypt-villus axis. Isolated epithelial cells scattered throughout the villus were, however, highly positive (Figure 4.5, panel B). The lamina propria, muscularis mucosae, and underlying submucosal regions were all free of specific signal (Figure 4.5, panel A; Figure 4.6, panel A). Control sections exhibited no specific signal (Figure 4.5, panels C and D; Figure 4.6, panels C and D).

Colonic epithelium did not exhibit specific signal in experimental tissue sections (Figure 4.7, panels A and B), nor was signal observed in the intestinal glands that could be distinguished from non-specific signal as seen in control sections (Figure 4.7, panels C and D). Specific signal was not observed in the muscularis mucosae, in the submucosa, or in the underlying smooth muscle layers (Figure 4.7, panels A and B).

In the lung, no signal was detected in the alveolar epithelium (Figure 4.8, panels A and B). Bronchial epithelium and smooth muscle observed within the lung sections

Figure 4.4. Bright field photomicrographs demonstrating in situ hybridization of a ^{35}S-labeled PG33 cRNA probe in porcine tracheal tissue sections. A: cross-section of trachea (x10) hybridized with antisense cRNA probe. B: tracheal epithelium and submucosa (x20) hybridized with antisense cRNA probe. C: increased magnification of tracheal epithelium hybridized with antisense cRNA probe. D: tracheal epithelium and submucosa (x20) hybridized with control (sense) cRNA probe.

A.



B.

C.

D.

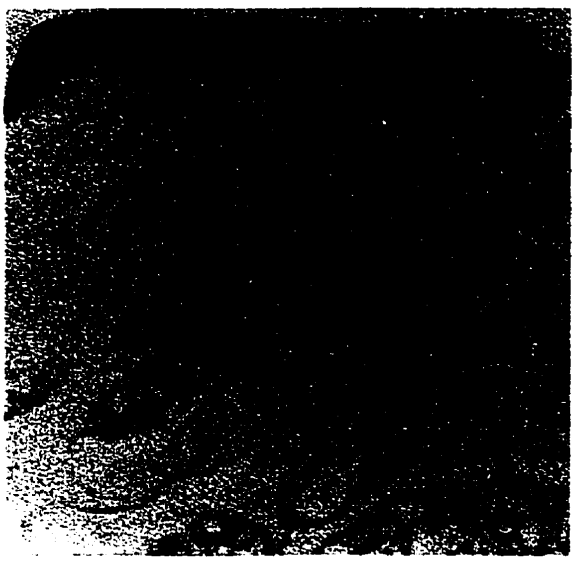
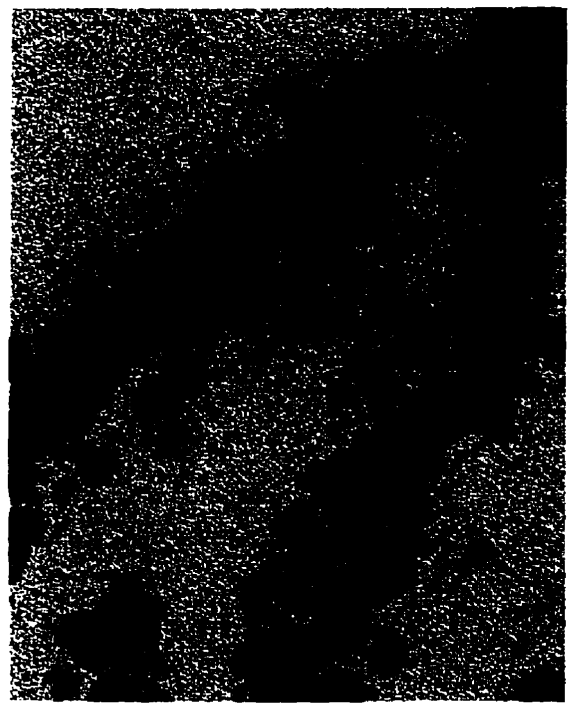


Figure 4.5. Bright field photomicrographs demonstrating in situ hybridization of a ^{35}S-labeled PG33 cRNA probe in villus region of porcine ileal tissue sections. A: cross-section of villus (x20) hybridized with antisense cRNA probe . B: increased magnification of villus section hybridized with antisense cRNA probe. C: cross-section of villus hybridized with control (sense) cRNA probe. D: increased magnification of villus section hybridized with sense cRNA probe.

A.

B.



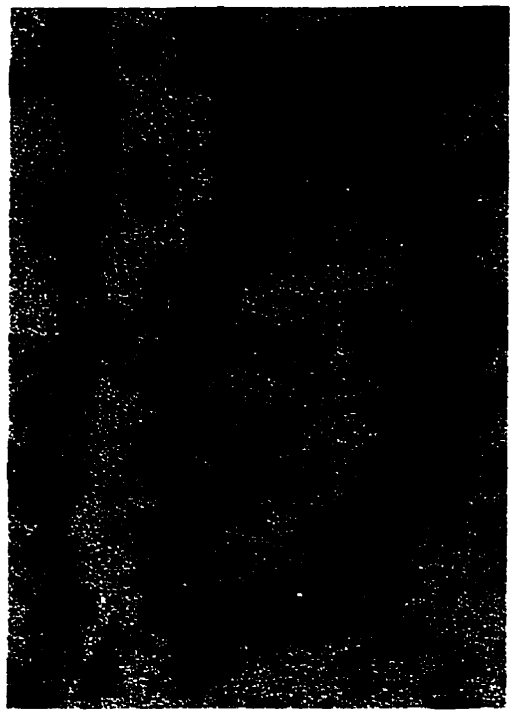
C.

D.

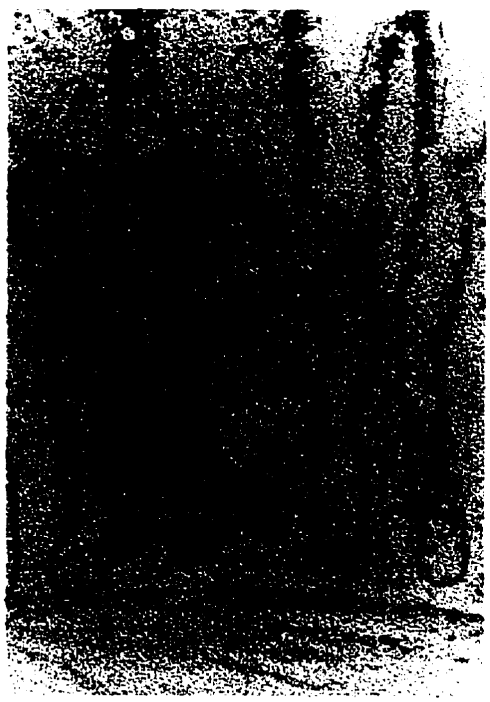
Figure 4.6: Bright field photomicrographs demonstrating in situ hybridization of a ^{35}S-labeled PG33 cRNA probe in crypt region of porcine ileal tissue sections. A: cross-section of crypt region (x20) hybridized with antisense cRNA probe. B: increased magnification of crypt region hybridized with antisense probe. C: cross-section of crypt region (x20) hybridized with control (sense) cRNA probe. D: increased magnification of crypt region hybridized with control probe.

A.

B.



C.



D.

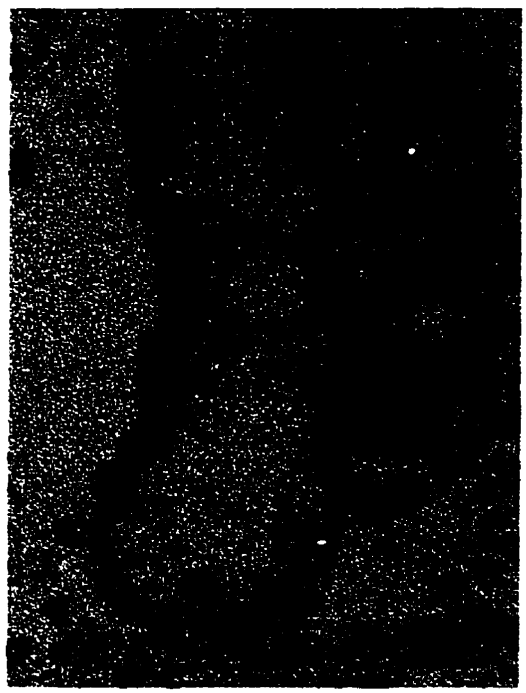
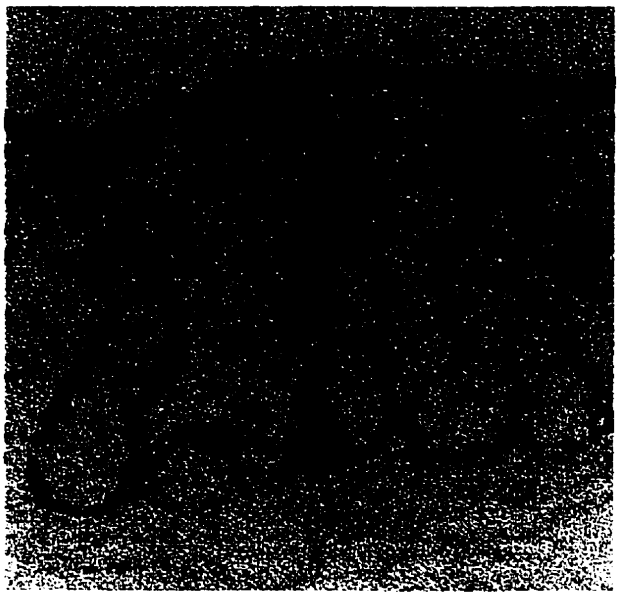


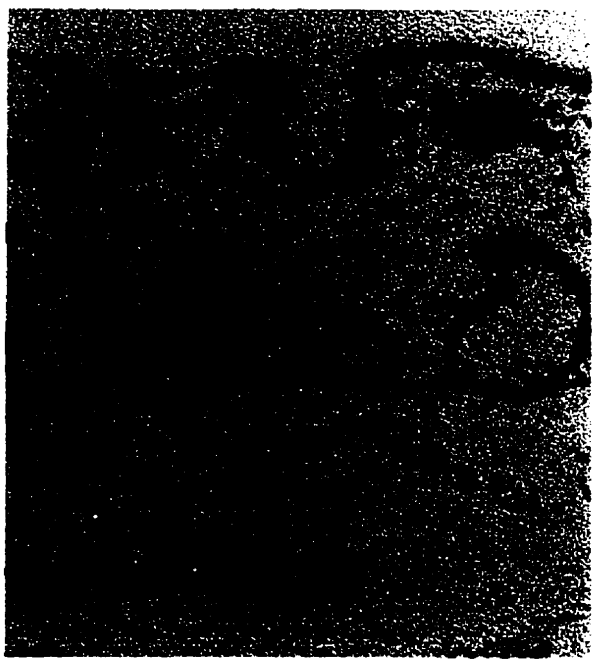
Figure 4.7. Bright field photomicrographs demonstrating in situ hybridization of a ^{35}S-labeled PG33 cRNA probe in porcine large intestinal tissue sections. A: cross-section of colonic epithelium and submucosa (x20) hybridized with antisense cRNA probe. B: increased magnification of colonic epithelium hybridized with antisense probe. C: colonic epithelium and submucosa (x20) hybridized with control (sense) cRNA probe. D: increased magnification of colonic epithelium hybridized with control probe.

A.



B.

C.



D.

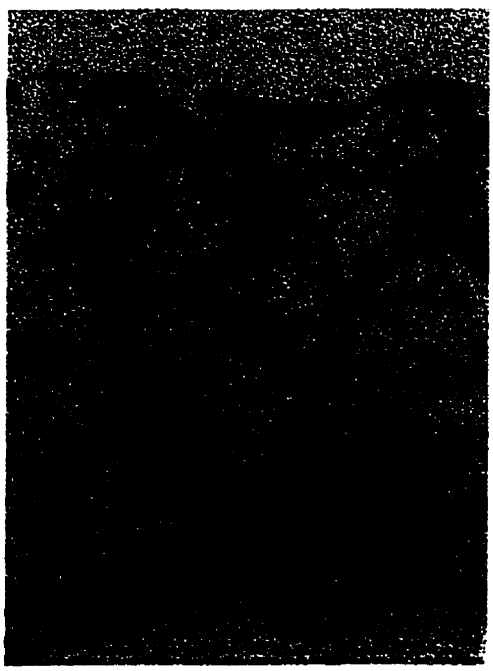
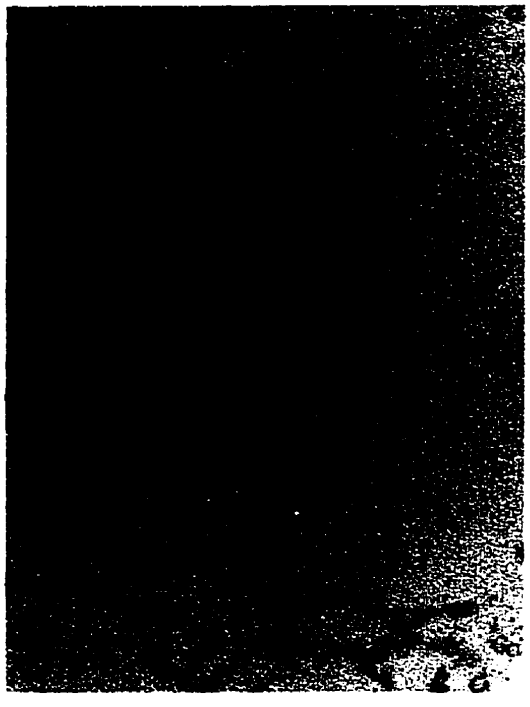


Figure 4.8: Bright field photomicrographs demonstrating in situ hybridization of a ^{35}S-labeled PG33 cRNA probe in porcine lung tissue sections. A: cross-section of lung, including alveoli and respiratory bronchioles (x10) hybridized with antisense cRNA probe. B: alveolar epithelium (x40) hybridized with antisense cRNA probe. C: alveolar epithelium (x20) hybridized with control (sense) cRNA probe.

A.

B.



C.

were also negative (Figure 4.8, panel A), as were control lung sections (Figure 4.8, panel C). No PG33 signal was detected in cardiac muscle fibers of porcine heart that could be distinguished from nonspecific signal in control sections (not shown). Likewise, while granule density at first glance appears to indicate signal in the pancreas, including exocrine acinar cells and intralobular and interlobular epithelial cells, background was consistently high in these sections and the signal is not specifically associated with cells (Figure 4.9, panels A and B). This signal was therefore considered to be non-specific.

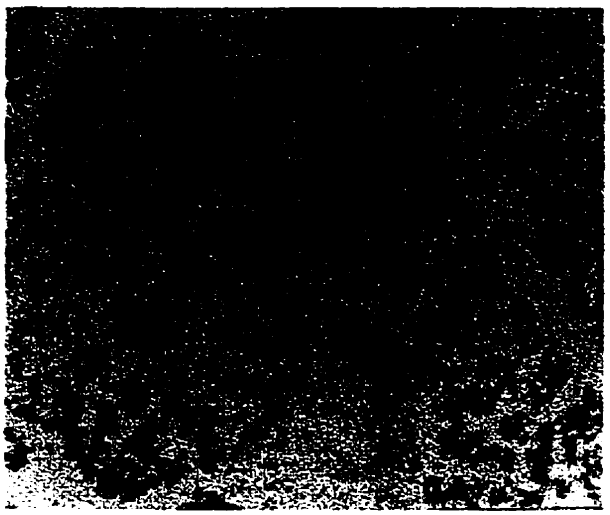
4.4 Discussion

Two complementary techniques have been used to investigate the tissue expression of PG33 message in the pig. A number of tissues were first examined through PCR amplification of PG33 internal sequences from reverse transcribed total cellular RNA. PG33 message was detected in small intestinal samples, the tissue from which PG33 cDNA had originally been isolated using an anti-chloride conductance monoclonal antibody. This confirms that PG33 mRNA is expressed in the same tissue that contains the antigen recognised by the monoclonal antibody . Furthermore, in situ hybridization identified the epithelium of the ileum as the specific cell type in which PG33 message is present. This is consistent with the original source of antigen against which the monoclonal antibody had been raised, which was purified ileal epithelial brush-border membrane protein.

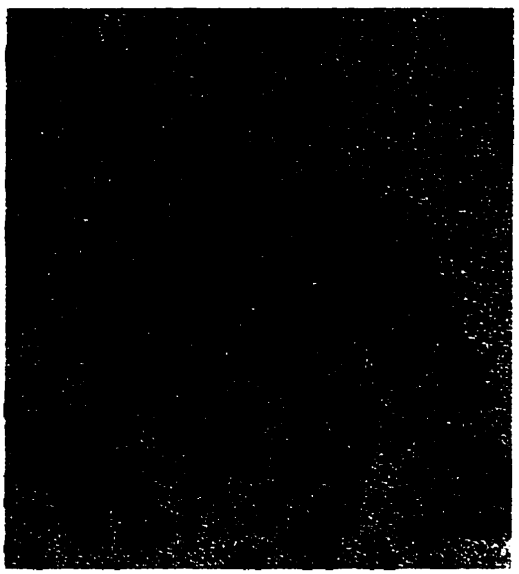
Previous immunohistochemical studies had also identified crypt epithelia as the intestinal cells expressing the protein antigen recognised by the monoclonal antibody (Racette *et al.*, 1996), though the level of resolution of those experiments was not sufficient to assign an apical or basolateral location to the antigen. The antibody used in these studies strongly inhibited conductive chloride uptake into apical membrane vesicles. Because transepithelial chloride movement drives fluid secretion into the gut lumen, and this occurs predominantly in the crypt regions (Welsh *et al.*, 1982), antibody recognition of a crypt antigen is consistent with a role for that antigen in the secretory

Figure 4.9: Bright field photomicrographs demonstrating in situ hybridization of a ^{35}S-labeled PG33 cRNA probe in porcine pancreatic tissue sections. A: cross-section of pancreas (x20) hybridized with antisense cRNA probe. B: increased magnification of pancreatic ductal epithelium hybridized with antisense cRNA probe. C: pancreatic ductal epithelium (x20) hybridized with control (sense) cRNA probe. D: increased magnification of pancreatic ductal epithelium hybridized with control probe.

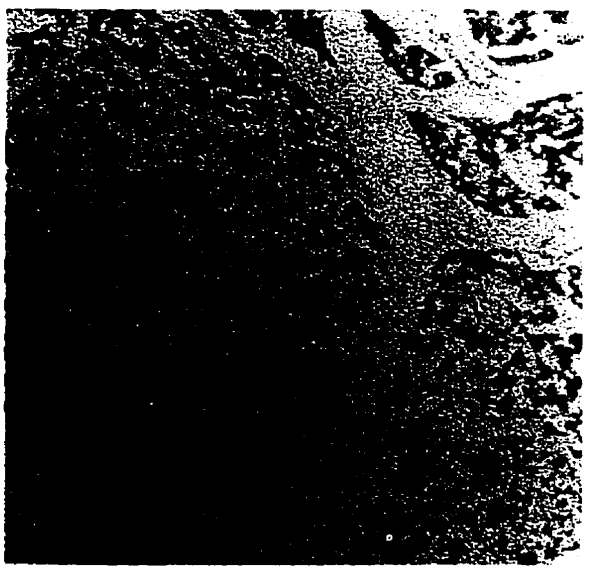
A.



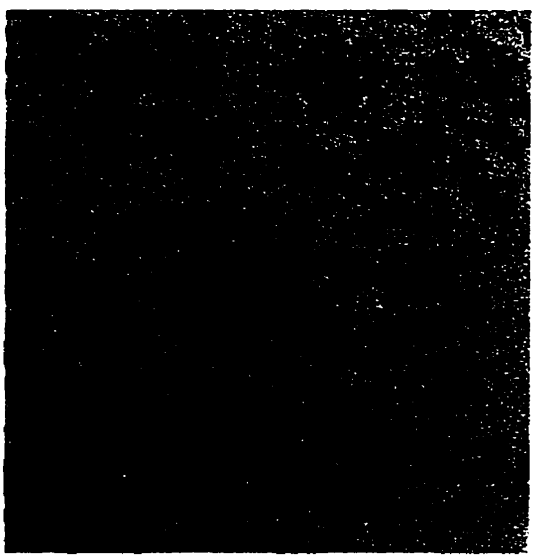
B.



C.



D.



process.

While the monoclonal bound most strongly in the intestinal crypts, PG33 message was detected in both the crypt and villus regions of the small intestine. Expression of mRNA in a cell does not, however, always correlate with the presence of the corresponding protein in that cell. In addition, the immunohistochemical studies had demonstrated some binding of the monoclonal to the villus epithelium (Racette *et al.*, 1996), but because the control tissue sections had also exhibited some villus signal, especially in the associated glycocalyx, definitive conclusions about the expression of the antigen in these cells were not made. Hence, expression of the PG33 gene product in the villus epithelium remains a possibility.

In addition to the diffuse signal seen throughout the crypt and villus surface epithelium, isolated cells within the villi exhibited a very strong signal, which presumably indicates a high level of PG33 transcript within these cells. These cells could not be identified as morphologically distinct from the other epithelial cells. The presence of scattered, strongly positive cells is not without precedent; human airway and small intestinal CFTR exhibits a similar pattern of expression in which moderate signal is seen throughout the surface epithelium, along with a small population of isolated cells with extremely high signal (Tresize & Buchwald, 1991; Engelhardt *et al.*, 1992; Strong *et al.*, 1994). Previous reports had not been able to assign identities to these cells with any degree of confidence, though investigators speculated that these cells may be specialised for some CFTR-associated function such as chloride secretion (Strong *et al.*, 1994). It is not known whether the cells expressing high levels of PG33 message are the same cells as those described in these reports.

In situ hybridization studies of selected other porcine tissues confirm the results of the RT PCR investigations in that specific PG33 message was detected in trachea but not colon, lung, pancreas, or heart. The expression of PG33 message may therefore be tissue-restricted, unlike that of CFTR, the ORCC, and ClC-2, all of which have a widespread tissue distribution. Other chloride channels with a tissue-specific expression pattern include the ClC-K subfamily, ClC-1, and the bovine calcium-

activated channel. While the ClC-K subfamily and ClC-1 are expressed predominantly in kidney and skeletal muscle (Steinmeyer *et al.*, 1991; Sakamoto *et al.*, 1996), respectively, the calcium-activated channel has been documented only in bovine trachea to date (Cunningham *et al.*, 1995). These channels, and by extension PG33, may be involved in tissue-specific processes, as opposed to more widely expressed channels; it has been suggested, for example, that ClC-2 may be important in the control of cell volume (Pusch & Jentsch, 1994).

In situ hybridization of a ^{35}S-labeled PG33 cRNA probe to tracheal tissue sections revealed that PG33 is expressed in this tissue in a cell-specific manner. Signal was detected in surface epithelial cells, which are thought to contribute to the thin fluid layer lining the surface of the airways (Welsh, 1987). The submucosal glands also exhibited extremely strong signal, indicative of high levels of PG33 transcripts in these cells, though it could not be determined whether the cells expressing PG33 were serous, mucous, or whether both cell types express PG33.

The tracheal expression of PG33 echoes that of CFTR, which is also found predominantly in the submucosal glands and, to a lesser extent, in the surface epithelium (Engelhardt *et al.*, 1992). CFTR is strongly expressed in the serous tubules of the submucosal glands, and in isolated cells along the collecting duct (Engelhardt *et al.*, 1992; Finkbeiner *et al.*, 1994). The serous tubules lie distal to the mucous tubules, and are thought to secrete a fluid which flushes the secreted mucus out of the tubules and into the collecting duct of the gland (Welsh, 1987). In CF, these glands become progressively blocked with accumulated mucus (Welsh *et al.*, 1995), presumably due to a defect in serous fluid secretion (Engelhardt *et al.*, 1992; Yamaya *et al.*, 1991). The concentration of PG33 message in the submucosal glands may indicate a similar physiological role for this gene product.

The only other tissues examined which were shown via RT PCR to express PG33 were the salivary glands. Parotid, mandibular, and sublingual glands all expressed varying levels of PG33 message. RT PCR does not allow a precise quantitation of mRNA present within a sample. Assuming, however, similar RNA

integrity and enzyme activity in all samples, PG33 expression appears to be lowest in the mandibular gland, moderate in the sublingual, and highest in the parotid gland.

The composition of salivary secretions from these glands varies according to species. In most species, including the pig, the parotid gland consists almost entirely of serous acini (Cook *et al.*, 1994). In the sublingual gland of the pig, on the other hand, the acinar endpieces are predominantly mucous (Dellmann, 1993). Cell composition varies according to species in the mandibular gland, and sexual dimorphism is moreover seen in some species, including pigs (Cook *et al.*, 1994). Serous cells are reported to predominate in males and mucous cells in females, though cells of both types can be seen in each gender (Cook *et al.*, 1994). This variation in cell type may explain the differences in PG33 expression in the three major salivary glands, though there is no immediately apparent pattern of distribution, except that expression is highest in the predominantly serous parotid gland. In situ hybridization studies were not conducted on salivary gland tissue; consequently, the identity of the salivary gland cells expressing high levels of PG33 is currently unknown.

To summarise, the expression of PG33 mRNA has been correlated with the location of the protein antigen recognised by the anti-chloride conductance monoclonal antibody in porcine small intestine. Of the porcine tissues investigated, PG33 was only detected in trachea and salivary gland in addition to the small intestine. While these epithelial tissues are secretory in nature, PG33 expression may not be a function of all secretory epithelia, since PG33 was not detected in exocrine pancreas, stomach, or large intestine. The PG33 message was not detected in liver, skeletal muscle, cardiac muscle, or kidney, nor was it detected elsewhere within the respiratory tract or pulmonary parenchyma. In both trachea and small intestine, PG33 was detected in surface epithelium; this transcript was furthermore detected in tracheal submucosal glands, a location in which CFTR is also highly expressed. These findings are consistent with a role for the PG33 gene product in the secretory process in the tissues in which it is expressed.

5.0 Comparison of the Tissue Expression of PG33 and the Bovine Chloride Channel Ca-CC

5.1 Introduction

Calcium-regulated chloride secretion functions as an important alternative pathway to the activation of chloride secretion via cAMP-dependent agonists. In some tissues, such as human sweat glands, the calcium-dependent pathway actually accounts for the majority of agonist-induced increases in chloride secretion (Sato & Sato, 1984; Reddy & Bell, 1996). In others, including human airways and murine pancreas, the magnitude of the two responses are nearly equal (Boucher *et al.*, 1989; Clarke *et al.*, 1994).

Under circumstances where cAMP-dependent chloride secretion is absent or defective, the calcium-regulated pathway may be upregulated. The *cftr*^{tm1Unc} (-/-) mouse, for example, shows an increased nasal epithelial response to ionomycin compared to normal littermates, indicating an upregulation of the calcium-activated pathway (Grubb *et al.*, 1994). A protective role has therefore been proposed for this alternative conductance in tissues affected by CF. Tissues in which this conductance can be demonstrated, such as pancreas and airways, are only mildly affected by the absence of functional CFTR in *cftr*^{tm1Unc} mice (Clarke *et al.*, 1994). In the small and large intestine, where this alternative conductance is absent, a much more severe disease phenotype manifests (Clarke *et al.*, 1994). Multiple chloride conductive pathways, then, are at the very least useful in preserving transepithelial chloride secretion, and may in some circumstances be essential.

In 1991, a group of investigators reported the isolation of a chloride channel from bovine tracheal epithelium (Ran and Benos, 1991). A candidate protein was

isolated when apical membrane vesicle preparations were subjected to a series of procedures involving detergent solubilization and anion and cation exchange chromatography. The fractions showing the highest specific activity in a ^{125}I-uptake assay correlated with the appearance of a 38 kDa protein species on SDS-PAGE; when this band was electroeluted and reconstituted into proteoliposomes, it continued to mediate the uptake of ^{125}I across the liposomal membrane. This activity was inhibited by the potassium ionophore valinomycin, which eliminated the electrical gradient across the liposomal membrane, and also by the anion channel blocker DIDS.

Because the specific activity of the eluted protein was enhanced only 12-fold compared to the total solubilized protein, milder isolation conditions were sought. Immunopurification using a polyclonal antibody raised against the 38 kDa species allowed a further 24-fold increase in specific activity as measured by the rate of ^{125}I uptake across reconstituted proteoliposomes (Ran and Benos, 1992).

Separation of the electroeluted protein on a polyacrylamide gel consistently demonstrated the presence of minor bands at 62, 64, and 140 kDa as well as the major band at 38 kDa (Ran and Benos, 1991, 1992). This was intriguing because the size range eluted was only 35-42 kDa. All of these bands were detected, however, by the anti-p38 antibody in a Western blot, leading to the hypothesis that the 62-64 kDa doublet and the 140 kDa singlet were somehow derived from the 38 kDa band (Ran and Benos, 1992).

Treatment of the total solubilized protein with the reducing agent dithiothreitol (DTT) caused the larger bands to disappear, leaving only the 38 kDa band (Ran and Benos, 1992). The most likely interpretation of these results was that the 140 kDa and the 62-64 kDa species are, respectively, tetrameric and dimeric forms of the protein, and that DTT treatment reduced the disulfide bonds linking the subunits, leaving only the 38 kDa monomer. Subsequent experiments appeared to confirm this hypothesis (Ran *et al*, 1992).

The single channel properties of p38 reconstituted into liposomes and planar lipid bilayers were investigated (Ran *et al*, 1992; Fuller *et al*, 1994). Based on its

reversal potential in symmetrical KCl, the membrane protein was found to be anion-selective, with an anion permeability sequence of $I^- > Br^- > Cl^-$. The single-channel conductance of the protein was 25-30 pS in symmetrical 150 mM KCl and the current-voltage relationship was linear. Channel activity was only seen when the 140 kDa protein was present; neither the 62-64 kDa species nor the 38 kDa monomer appeared to function as a channel.

The native 140 kDa protein fused with planar lipid bilayers does not respond to PKA and ATP or to ATP alone, nor is it phosphorylated *in vitro* by PKA and ATP (Fuller *et al*, 1994). The protein is phosphorylated *in vitro* by CaMK II, and while the channel does not respond to physiological-range increases in Ca^{2+} alone, the addition of 0.5 to 1 μM Ca^{2+} to the bath solution after the channel has been phosphorylated by CaMK II causes an increase in channel activity in planar lipid bilayers (Fuller *et al*, 1994). High, nonphysiological levels of Ca^{2+} (above 3 μM) can also activate the channel (Fuller *et al*, 1994).

The anti-p38 polyclonal antibody was used to screen a bovine tracheal expression library for the cDNA encoding the p38 protein. The full-length cDNA subsequently isolated was 3001 bp long, with an open reading frame from bases 19 to 2730 (Cunningham *et al*, 1995). This would code for a protein containing 903 amino acids with a predicted molecular mass of approximately 100 kDa, far larger than the 38 kDa species used to raise the screening antibody.

A labeled segment of the cDNA clone (now called Ca-CC) hybridized to a 3.1 kb transcript in a Northern blot performed on bovine tracheal mRNA, which is in agreement with the length of the cloned cDNA (Cunningham *et al*, 1995). Amplification of internal sequences by RT-PCR confirmed the expression in bovine trachea, but showed no expression of the gene in bovine liver, lung, brain, or renal papillae (Cunningham *et al*, 1995). Ca-CC appears to be conserved between at least some species, as the cDNA probe cross-hybridized to mouse, rat, dog, rabbit, monkey, and human genomic DNA, though not to yeast or chicken DNA (Cunningham *et al*, 1995).

Analysis of the predicted amino acid sequence of Ca-CC shows the presence of multiple protein kinase recognition sequence motifs. While there are only two consensus sequences for protein kinase A and three for tyrosine kinase, there are 10 for CaMK II and 15 for protein kinase C (Cunningham *et al*, 1995). These potential sites may not all serve as phosphoacceptors, since earlier experiments had shown Ca-CC is not responsive to PKA and ATP, though no *in vitro* or *in vivo* studies of *in vitro* translated Ca-CC phosphorylation have been reported.

The major product of *in vitro* translation was a 100 kDa protein; when translated in the presence of pancreatic microsomes, which allows glycosylation of the core protein, the major product migrated at 140 kDa on SDS-PAGE (Cunningham *et al*, 1995). However, neither the 100 nor the 140 kDa products could be reduced by treatment with DTT, unlike the immunopurified 140 kDa protein (Cunningham *et al*, 1995). Antibodies raised against a Ca-CC fusion protein did recognise the 38 kDa protein in a Western blot on bovine tracheal membrane vesicles (Cunningham *et al*, 1995).

Expression of Ca-CC in COS-7 cells correlated with the appearance of a linear, anion-selective current in the presence of the calcium ionophore ionomycin (Cunningham *et al*, 1995). This increase in whole-cell current was inhibited by the addition of DTT. Likewise, injection of Ca-CC sense cRNA into *Xenopus* oocytes caused an increase in whole-cell current that was partially inhibited by both DIDS and by DTT (Cunningham *et al*, 1995). The current-voltage relationship in oocytes, however, was outwardly rectifying, unlike the channel activity in COS-7 cells. When vesicles from these Ca-CC-injected oocytes were fused with planar lipid bilayers, a calcium-regulated channel activity appeared that had a single-channel conductance of 21 pS. This channel was also sensitive to DIDS and to DTT.

Certain inositol phosphate metabolites have been shown to act as signalling molecules in a variety of cell types (Barrett, 1997). In particular, D-*myo*-inositol 3,4,5,6-tetrakisphosphate [Ins(3,4,5,6)P_4] has been reported to inhibit calcium-activated chloride secretion in epithelial cells. This isomer was shown to inhibit Ca-CC activity

in planar lipid bilayers (Ismailov *et al*, 1996), which suggests the possibility of a direct interaction of Ins(3,4,5,6)P_4 with the channel.

The role of Ca-CC in airway fluid secretion has yet to be explored, but since the calcium-activated current is an essential component of net airway chloride secretion, Ca-CC may prove to be physiologically relevant in this tissue. Ca-CC is also the only DNA sequence with known homology to the cDNA clone PG33. While PG33 was isolated from porcine small intestine, Ca-CC appears confined to bovine trachea. The studies on Ca-CC tissue expression were not exhaustive, however, and it was thought important to investigate whether Ca-CC might also be present in tissues which are known to express PG33 and which were not studied in the original reports. In addition, the ZooBlot performed using Ca-CC as a probe in the original study did not include porcine genomic DNA. It was also of interest, therefore, to discover if Ca-CC is expressed in pig tissues, and if PG33 is expressed in bovine tissues. Reverse transcriptase PCR using gene-specific primers was the simplest and most sensitive technique which would allow all of these questions to be answered.

5.2 Methods

5.2.1 Cell Culture

Both T84 and HBE1 cells were a generous gift of Dr. Sherif Gabriel (University of North Carolina-Chapel Hill).

T84 human colonic carcinoma cells were grown in a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium (DME) containing 5% fetal bovine serum (FBS). Cells were maintained at 37°C in 95% O_2/5% CO_2. Cells were fed every three days, and passaged every 4 weeks.

Human bronchial epithelial cells (HBE1) were grown in Ham's F12+7X medium (supplemented with 10 μg/mL insulin, 10^{-6} M hydrocortisone, 3.75 μg/mL endothelial cell growth supplement (ECGS), 25 ng/mL epidermal growth factor (EGF), 3 x 10^{-8} M triiodothyronine, 5 μg/mL transferrin, and 10 ng/mL cholera toxin.) Cells were maintained at 37°C in 95% O_2/5% CO_2 and fed every three days. Polarized

differentiation was induced by supplementing the medium 1:1 with DME containing 2% FBS which had previously been conditioned for 3 days in the presence of confluent human 3T3 fibroblasts.

5.2.2 RNA Purification

Postmortem tissue samples were taken from weanling pigs of mixed Yorkshire-Landrace breeding (obtained from the Prairie Swine Centre), and Jersey (obtained from WCVM Necropsy) or Holstein (through kind permission of Dr. Germain Nappert) cows. Total RNA was purified as earlier described (Chomczynski and Sacchi, 1987), then treated with 10 units DNase I/μg RNA to remove any contaminating genomic DNA. DNase I was removed by extraction with one volume of phenol:chloroform, followed by ethanol precipitation of the RNA sample.

In the case of cultured cells, poly A+ RNA was directly purified using a QuickPrep Micro mRNA purification kit (Pharmacia) as previously described.

5.2.3 Reverse Transcriptase PCR

Samples were reverse transcribed using either the bovine [CaCl: 5' TTTAGGATTTATTTACAAACGG 3'] antisense primer or the porcine [105a: 5' AGCACGTCTTTGAGATTTTACG 3'] antisense primer. The reverse transcription was carried out under conditions identical to those described earlier, with the single exception that the SuperScript II reverse transcriptase enzyme (GibcoBRL) was used instead of the MMLV-reverse transcriptase enzyme.

PCR amplification of porcine sequences (PG33) was also identical to protocols previously described. The sense primer used to amplify PG33 was 920s [5' ACGATGCAAATGGTCGATACAG 3'], and the antisense primer was 105a [5' AGCACGTCTTTGAGATTTTACG 3']. The 838-base pair product generated under these reaction conditions could be visualised after 30 cycles of PCR amplification of cDNA from porcine tissue samples, and after 55 cycles if the cDNA had been reverse transcribed from T84 or HBE1 mRNA.

Amplification of the 766-base pair internal bovine Ca-CC sequence was carried

out under similar reaction conditions, with two exceptions. First, because the bovine antisense PCR primer, CaCl [5' TTTAGGATTTATTTACAAACGG 3'], has a lower melting temperature than the porcine antisense PCR primer 105a, the annealing step of the reaction was carried out at 45°C instead of 52°C. Second, the Ca-CC product could not be visualised after 30 cycles of amplification. One μL of the first, 30-cycle round was therefore reamplified under the same conditions as the first round for a further 25 cycles. The sense primer for PCR amplification of the Ca-CC product was 920s.

Internal controls were performed on 5 μg total RNA samples reverse transcribed with a β-actin-specific antisense primer [5' TAGATGGGCACAGTGTGGG 3']; PCR amplification of β-actin internal sequences was performed with the antisense primer and a β-actin-specific sense primer [5' GGCGTGATGGTGGGCATGG 3']. Amplification conditions were as described above, except that a 54°C annealing temperature was used. Products were visible after 30 cycles of PCR amplification.

All PCR products were visualised on 0.8% agarose gels containing 0.33% ethidium bromide as earlier described.

5.3 Results

Expression of PG33 appears to be restricted to secretory epithelial tissues such as the small intestine, the large airways, and the salivary glands. It is not, however, expressed in all secretory epithelia, as evidenced by the lack of PG33 message in exocrine pancreas. The bovine calcium-activated channel Ca-CC, on the other hand, could be detected in bovine trachea, but none of the other bovine tissues originally screened (Cunningham *et al*, 1995). The investigators did not report the expression of Ca-CC in the tissues in which PG33 is strongly expressed.

Reverse transcriptase PCR was performed on total cellular RNA from porcine ileum, parotid, and tracheal epithelia, and from bovine ileum, parotid, and tracheal samples. Gene-specific primers were designed to allow selective amplification of either the porcine PG33 message or the bovine Ca-CC message from a given sample. A template that had been reverse transcribed with a bovine primer could not be PCR-

amplified with the porcine gene-specific primers, nor could porcine reverse-transcribed templates be PCR-amplified with the bovine gene-specific primers. The porcine PCR primers were designed to amplify an 838-base pair internal segment of PG33 corresponding to bases 1355-2193, and the bovine primers amplified a 766-base pair internal Ca-CC sequence between bases 2030 and 2796 of the bovine cDNA.

The expression of Ca-CC in bovine trachea was confirmed (see Figure 5.1, lane 2), demonstrating that our system can detect Ca-CC message as well as PG33 message. Amplification of the Ca-CC internal sequences required a minimum of 2 rounds of PCR, and a total of 55 cycles of amplification. Ca-CC expression was not detectable in bovine parotid or ileal samples (Figure 5.1, lanes 4 and 6), nor could PCR products be amplified by the use of PG33-specific PCR primers on CaCl-reverse transcribed templates from these tissues (Figure 5.1, lanes 3, 5, and 7). Integrity of the bovine RNA samples was demonstrated by the amplification of an internal sequence of a housekeeping gene, β-actin (Figure 5.1C).

Samples of tracheal, parotid, and ileal epithelia were taken from pig and screened for expression of Ca-CC. Expression of the bovine gene was not detected in any of these porcine tissues (Figure 5.2, lanes 2, 4, and 6). Total RNA from the porcine tissues was intact, however, since β-actin-specific sequences could be amplified from these samples (Figure 5.2C). Several small DNA fragments under 200 bp in size were amplified from the porcine samples, but there was no detectable expression of the 766-base pair product specific to Ca-CC. These small fragments were therefore considered to be nonspecific artifacts. Amplification of cDNA reverse transcribed with CaCl using PG33-specific PCR primers did not result in any discernable products (Figure 5.2, lanes 3, 5, and 7).

The same total cellular RNA samples were used to assess the expression of PG33. The presence of the PG33 message in porcine ileum, parotid, and trachea was confirmed (see Figure 5.3, lanes 2, 4, and 6), in agreement with earlier studies (see Chapter 4: Tissue Expression). Unlike the Ca-CC product, the PG33 internal product could be amplified during a single round, 30 cycles in total, of PCR. cDNA reverse

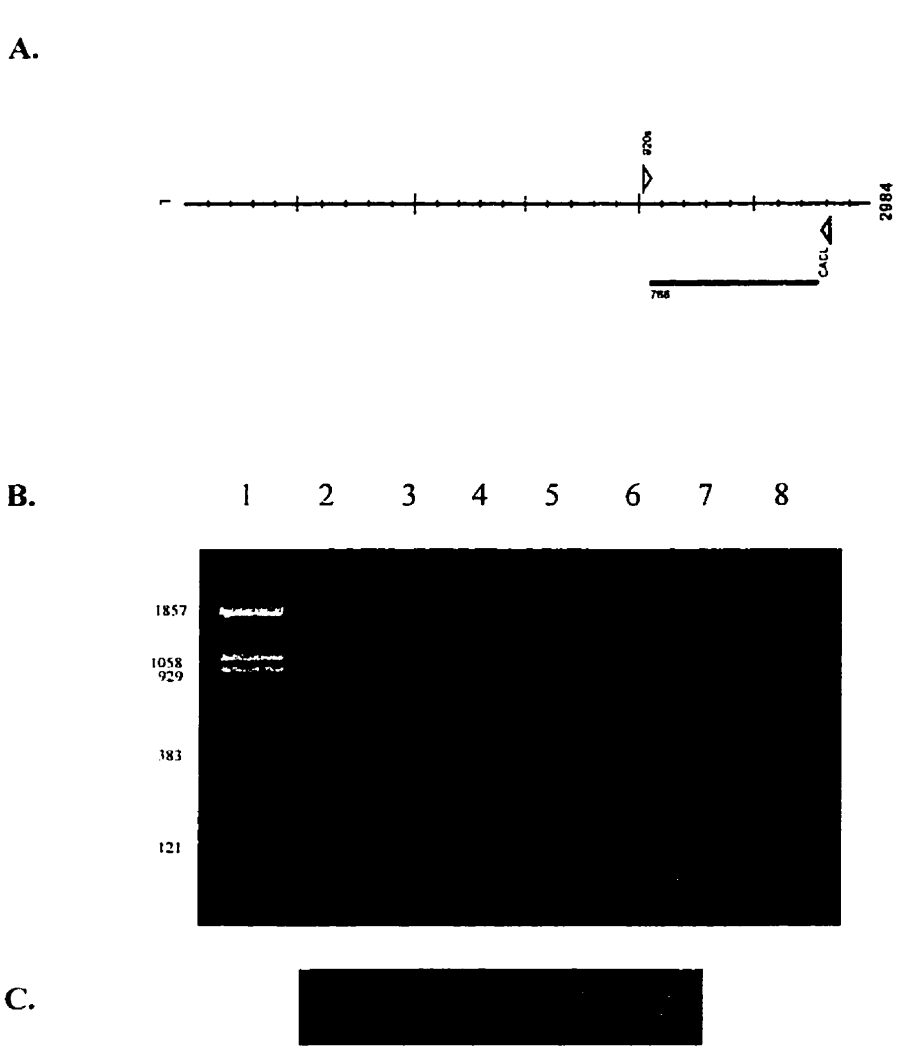


Figure 5.1. Expression of Ca-CC in bovine trachea, ileum, and parotid gland. A: schematic depicting amplification of 766-bp internal Ca-CC sequence. B: Five μg total RNA was reverse transcribed with the CaCl primer. cDNA was PCR-amplified for two rounds, a total of 55 cycles, with the sense primer 920s and either the Ca-CC internal antisense primer CaCl or the PG33 internal antisense primer105a. Lane 1: pBR322-BstN I standards (1857, 1058, 929, 383, and 121 bp). Lane 2: bovine tracheal cDNA amplified with Ca-CC-specific primers. Lane 3: bovine tracheal cDNA amplified with PG33-specific primers. Lane 4: bovine ileal cDNA amplified with Ca-CC-specific primers. Lane 5: bovine ileal cDNA amplified with PG33-specific primers. Lane 6: bovine parotid cDNA amplified with Ca-CC-specific primers. Lane 7: bovine parotid cDNA amplified with PG33-specific primers. Lane 8: no template control. C: β-actin internal controls.

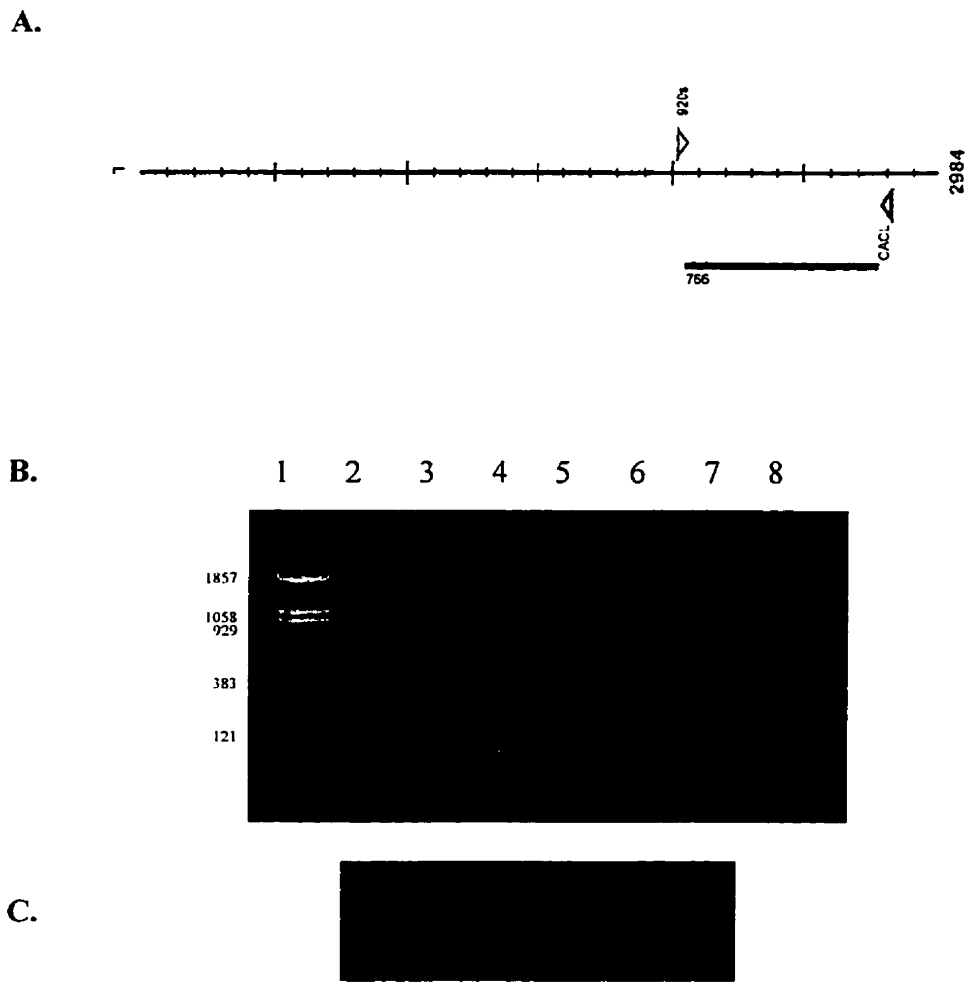


Figure 5.2. Expression of Ca-CC in porcine trachea, ileum, and parotid gland. A: schematic depicting amplification of Ca-CC 766-bp product. B: Five μg total RNA was reverse transcribed with the CaCl primer. cDNA was PCR-amplified with the sense primer 920s and either the Ca-CC-specific antisense primer CaCl or the PG33-specific antisense primer 105a for 2 rounds of PCR, a total of 55 cycles. Lane 1: pBR322-BstN I standards (1857, 1058, 929, 383, and 121 bp). Lane 2: porcine tracheal cDNA amplified with Ca-CC-specific primers. Lane 3: porcine tracheal cDNA amplified with PG33-specific primers. Lane 4: porcine ileal cDNA amplified with CaCC-specific primers. Lane 5: porcine ileal cDNA amplified with PG33-specific primers. Lane 6: porcine parotid cDNA amplified with Ca-CC-specific primers. Lane 7: porcine parotid cDNA amplified with PG33-specific primers. Lane 8: bovine tracheal cDNA amplified with Ca-CC-specific primers (positive control). C: β-actin internal controls.

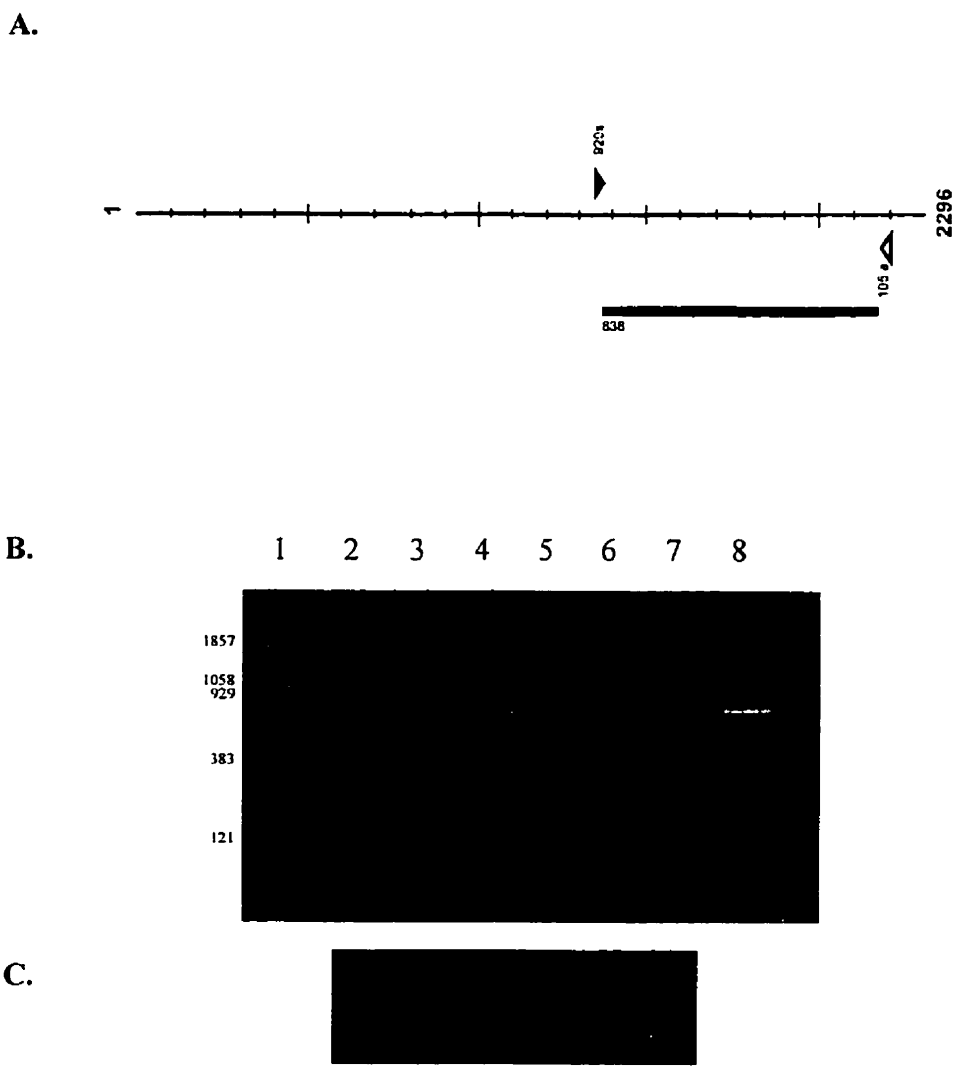


Figure 5.3. Expression of PG33 in porcine trachea, ileum, and parotid gland. A: Schematic indicating amplification of 838-bp internal PG33 sequence. B: Five μg total RNA was reverse transcribed with the105a primer. cDNA was PCR-amplified with the sense primer 920s and either the PG33-specific antisense primer 105a for 30 cycles, or the Ca-CC-specific antisense primer CaCl for 55 cycles. Lane 1: pBR322-BstN I standards (1857, 1058, 929, 383, and 121 bp). Lane 2: porcine tracheal cDNA amplified with PG33-specific primers. Lane 3: porcine tracheal cDNA amplified with Ca-CC-specific primers. Lane 4: porcine ileal cDNA amplified with PG33-specific primers. Lane 5: porcine ileal cDNA amplified with Ca-CC-specific primers. Lane 6: porcine parotid cDNA amplified with PG33-specific primers. Lane 7: porcine parotid cDNA amplified with Ca-CC-specific primers. Lane 8: PG33 amplified with PG33-specific primers (positive control). C: β-actin internal controls.

transcribed with the PG33-specific antisense primer could not be PCR-amplified with primers specific for Ca-CC (Figure 5.3, lanes 3, 5, and 7).

PG33 message could not be demonstrated in bovine ileum, parotid, or trachea (Figure 5.4, lanes 2, 4, and 6), nor, if the cDNA had been reverse transcribed with the PG33-specific antisense primer, did PCR performed with the Ca-CC primers result in the amplification of the Ca-CC product (Figure 5.4, lanes 3, 5, and 7).

The presence of Ca-CC protein in the colonic epithelial cell line T84 has been demonstrated by immunoblot (Ran and Benos, 1992), and it has been suggested that Ca-CC may contribute to a calcium-regulated chloride conductance in these cells (Ran and Benos, 1992; Morris & Frizzell, 1994; Barrett, 1997). It was of interest, therefore, to compare the potential gene expression of Ca-CC and PG33 in this cell line.

Reverse transcriptase PCR shows that PG33 message can be detected in undifferentiated T84 cells, although amplification of specific product required 55 PCR cycles, rather than the 30 cycles necessary to amplify this product from porcine tissue samples (Figure 5.5, lane 3). The bovine CaCC message can also be detected in undifferentiated T84 cells (Figure 5.5, lane 6), confirming the observations of earlier reports (Ran and Benos, 1992). The product amplified with CaCC-specific primers, however, contained several additional DNA products besides the target sequence. While some of these products were smaller than the expected 766-base pair product, one product was much larger, approximately 1600 base pairs in size. None of these products had been observed earlier during the amplification of Ca-CC from bovine tracheal cDNA.

Because of these unexpected results in T84 cells, another human cell line was investigated for Ca-CC and PG33 expression. The HBE1 human bronchial epithelial cell line is known to express both cAMP-dependent and Ca^{2+}-regulated chloride conductances (Yankaskas *et al.*, 1993). Like T84 cells, HBE1 cells also express both PG33 and Ca-CC, but at very low copy numbers (Figure 5.6). Fifty-five cycles of PCR over two rounds were required to amplify both the PG33 and Ca-CC internal sequences from HBE1 cDNA.

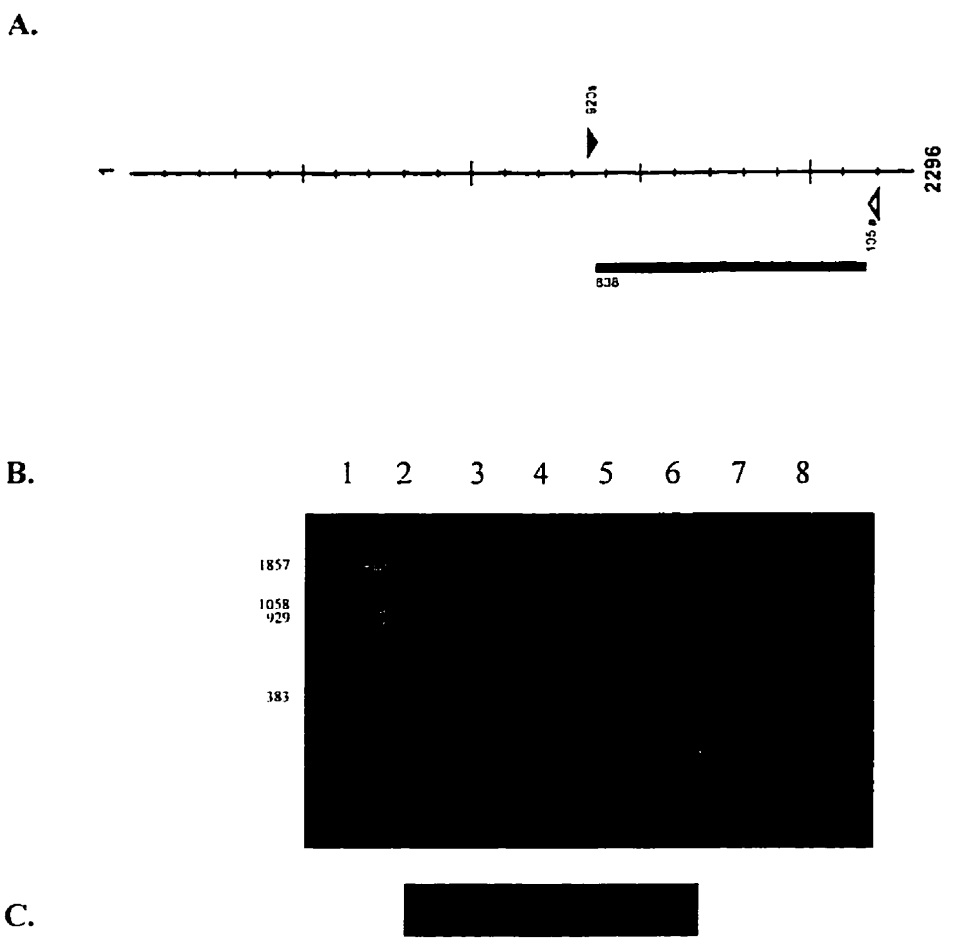


Figure 5.4. Expression of PG33 in bovine trachea, ileum, and parotid gland. A: Schematic indicating amplification of internal PG33 sequence. Five μg total RNA was reverse transcribed with the 105a primer. cDNA was PCR-amplified with the sense primer 920s and either the PG33-specific antisense primer 105a for 30 cycles or the Ca-CC-specific antisense primer CaCl for 55 cycles. B: Lane 1: pBR322-BstN I standards (1857, 1058, 929, 383, and 121 bp). Lane 2: bovine tracheal cDNA amplified with PG33-specific primers. Lane 3: bovine tracheal cDNA amplified with Ca-CC-specific primers. Lane 4: bovine ileal cDNA amplified with PG33-specific primers. Lane 5: bovine ileal cDNA amplified with Ca-CC-specific primers. Lane 6: bovine parotid cDNA amplified with PG33-specific primers. Lane 7: bovine parotid cDNA amplified with Ca-CC-specific primers. Lane 8: PG33 amplified with PG33-specific primers (positive control). C: β-actin internal controls.

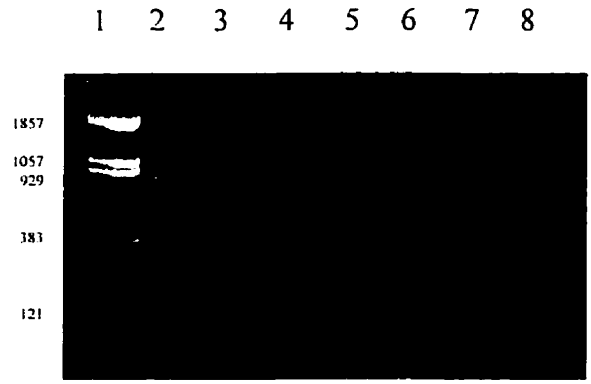


Figure 5.5. Expression of PG33 and Ca-CC in undifferentiated cells from the T84 intestinal epithelial cell line. One µg poly A+ RNA was reverse transcribed with either the PG33-specific primer 105a (lanes 2-4) or the Ca-CC-specific primer CaCl (lanes 5-7). cDNA reverse transcribed with 105a was PCR-amplified for 55 cycles with the antisense primer 105a and the sense primer 920s. cDNA reverse transcribed with CaCl was PCR-amplified for 55 cycles with the antisense primer CaCl and the sense primer 920s. Lane 1: pBR322-BstN I standards (1857, 1058, 929, 383, and 121 bp). Lane 2: PG33 amplified with PG33-specific primers (positive control). Lane 3: T84 cDNA amplified with PG33-specific primers. Lane 4: T84 polyA+ RNA amplified with PG33-specific primers (genomic DNA negative control). Lane 5: bovine trachea amplified with Ca-CC-specific primers (positive control). Lane 6: T84 cDNA amplified with Ca-CC-specific primers. Lane 7: T84 polyA+ RNA amplified with Ca-CC-specific primers (genomic DNA negative control). Lane 8: no template control.

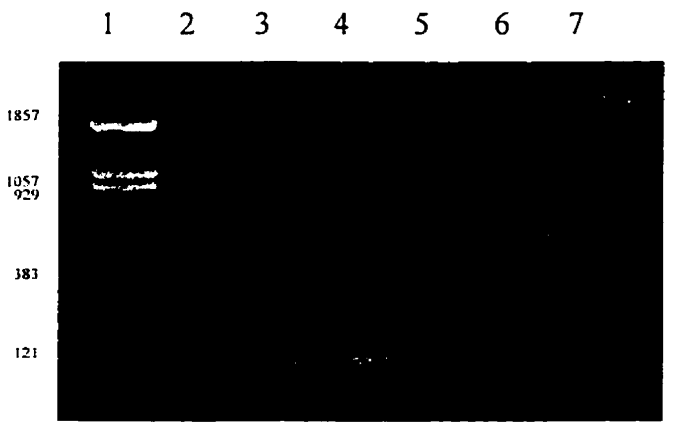


Figure 5.6. Expression of PG33 and Ca-CC in differentiated and undifferentiated HBE1 human airway epithelial cells. One μg poly A+ RNA was reverse transcribed with either the Ca-CC-specific antisense primer CaCl (lanes 2-3) or the PG33-specific antisense primer 105a (lanes 5-6). cDNA reverse transcribed with CaCl was PCR-amplified for 55 cycles with the sense primer 920s and the antisense primer CaCl. cDNA reverse transcribed with 105a was PCR-amplified for 55 cycles with the sense primer 920s and the antisense primer 105a. Lane 1: pBR322-BstN I (1857, 1058, 929, 383, 121 bp). Lane 2: undifferentiated HBE1 cDNA amplified with Ca-CC-specific primers. Lane 3: differentiated HBE1 cDNA amplified with Ca-CC-specific primers. Lane 4: bovine tracheal cDNA amplified with CaCC-specific primers (Ca-CC positive control). Lane 5: undifferentiated HBE1 cDNA amplified with PG33-specific primers. Lane 6: differentiated HBE1 cDNA amplified with PG33-specific primers. Lane 7: porcine ileal cDNA amplified with PG33-specific primers (PG33 positive control).

In addition to the expected 766-base pair product, the same larger product was observed when undifferentiated HBE1 mRNA was reverse transcribed with CaCl, then PCR-amplified with primers specific for Ca-CC (Figure 5.6, lane 2). When the template cDNA was reverse transcribed from mRNA purified from differentiated HBE1 cells, however, only the target 766-base pair sequence was visible (Figure 5.6, lane 3). The quantity of product amplified from differentiated HBE1 cells was also diminished compared to that amplified from undifferentiated cells.

Amplification of PG33 internal sequences from 105a-reverse transcribed cDNA from undifferentiated HBE1 cells resulted in multiple products, up to and including the 838-base pair expected product (Figure 5.6, lane 5). None of these products were present in large quantities. The same PCR reaction carried out on cDNA from differentiated HBE1 cells also resulted in multiple products, but in this case the predominant product was the 838-base pair target sequence (Figure 5.6, lane 6).

To summarise, expression of the calcium-regulated chloride channel Ca-CC was not detected in bovine tissues other than in tracheal epithelium. Expression of this gene could not be shown in other bovine secretory epithelia, such as the small intestine or the parotid salivary gland, nor was Ca-CC detected in any of these epithelia in pigs. PG33 message, while expressed in porcine trachea, ileum, and parotid, was not detected in bovine trachea, ileum, or parotid. Both of these genes, however, appear to be expressed in the human cell lines T84 and HBE1. In the case of HBE1 cells, expression of PG33 and Ca-CC may depend upon the differentiation status of the cell. This may also be true of the expression of these genes in T84 cells, but this could not be confirmed as T84 mRNA from differentiated cells was not available.

5.4 Discussion

The bovine calcium-activated chloride channel Ca-CC is the only cDNA currently known with significant nucleotide and amino acid homology to the PG33 cDNA. Because PG33 may also be involved in the epithelial chloride secretory process, it was considered important to investigate whether the pattern of tissue distribution of

mRNA for the two genes is similar or complementary.

In bovine tissues, the Ca-CC gene product can only be detected in trachea, the tissue in which it was first characterised. The original investigators reported that Ca-CC is not expressed in bovine brain, lung, liver, or kidney (Cunningham *et al.*, 1995). Surprisingly, however, none of the other characteristically secretory epithelia tissues such as intestine or pancreas were examined. In addition, while the presence of the Ca-CC gene was demonstrated in a number of species, genomic DNA from pig was not included in the Southern blot (Cunningham *et al.*, 1995). Several points therefore required clarification in order to assess a possible relationship between the bovine Ca-CC and the porcine PG33 gene product.

Antisense primers for the reverse transcription reactions were designed from the 3' untranslated regions of Ca-CC and PG33 (see Figure 5.7). The CaCl antisense primer bound specifically to the bovine Ca-CC cDNA, but was selected from a region of Ca-CC which had very little sequence homology to PG33. Likewise, the 105a antisense primer bound with high fidelity to PG33 cDNA, but was chosen from an area with low homology to Ca-CC. Used separately, then, these two sequences should specifically prime the reverse transcription of only Ca-CC or PG33 mRNA, but not both. This was confirmed by a series of control PCR reactions in which PG33-specific primers were used on a cDNA template reverse transcribed with the bovine CaCl primer. A parallel set of reactions were carried out in which Ca-CC-specific primers were used in the PCR amplification of a cDNA template which had been reverse transcribed with the porcine 105a primer. In neither case was product of the appropriate size obtained. Hence, PCR reactions were amplifying one cDNA or the other, but not both if only one antisense primer was present in the same reaction.

Reverse transcriptase PCR using the Ca-CC-specific primers confirmed the expression of Ca-CC in bovine tracheal epithelia. The standard 30-cycle PCR reaction conditions were, however, insufficient to amplify a visible product. Instead, it was necessary to amplify an aliquot of the first 30-cycle round for a subsequent 25 cycles before the 766-base pair internal sequence could be visualised on an agarose gel. Benos'

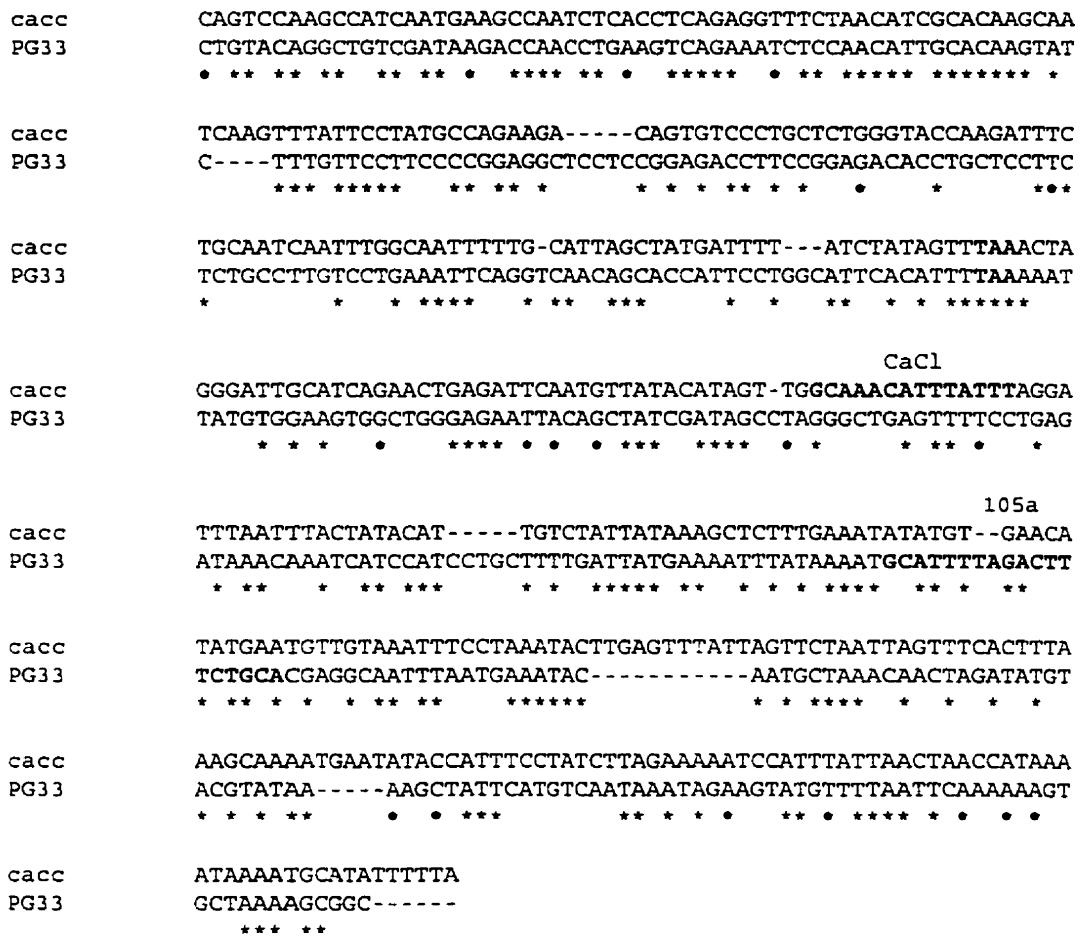


Figure 5.7. Primer binding sites for reverse transcriptase PCR of PG33 and Ca-CC. Stop codons, the PG33-specific antisense primer 105a, and the Ca-CC-specific antisense primer CaCl are in bold type. Asterisks indicate nucleotides that are identical in the two sequences.

group reported successful amplification after 30 cycles of PCR (Cunningham *et al.*, 1995), but these reactions required 200 ng of template cDNA. Despite the fact that the reverse transcription reaction was primed in their case with oligo-dT rather than a gene-specific primer, multiple reactions must have been pooled for a successful PCR amplification under the conditions described in this report. Taken together, these results suggest that the Ca-CC message is expressed in tracheal epithelial cells in an extremely low copy number. As a comparison, the PG33 message, which is estimated to occur in small intestinal cells at a frequency of 0.01% or less, can be amplified from porcine tissues after 30 rounds of PCR.

Even under the reaction conditions which successfully amplified the Ca-CC message from bovine trachea, this message could be demonstrated in neither bovine ileum nor parotid salivary gland. The tissue distribution of Ca-CC is clearly different, therefore, from that of PG33, which is expressed in porcine ileum and parotid as well as in porcine trachea. This may indicate that Ca-CC is important to some specialised process of tracheal epithelial cells, while the PG33 gene product may be involved in a function common to several types of secretory epithelial cells.

The Ca-CC and the PG33 gene may not be entirely conserved between species. Ca-CC cross-hybridizes with genomic DNA from rabbit, dog, mouse, rat, monkey, and human as well as cow, but not to DNA from yeast or chicken (Cunningham *et al.*, 1995). Under these experimental conditions, Ca-CC message could not be demonstrated in porcine tissues, including ileum and parotid as well as trachea. Likewise, PG33 message could not be amplified by PCR from RNA pools from any of the bovine tissues investigated. While the tissue expression of the two genes in their respective species appears to be different, this lack of conservation between the two species may indicate that the two genes play similar roles in the tissues in which they are expressed.

An alternative possibility exists that may explain the apparent lack of conservation of these genes between cattle and pigs. The antisense primers were selected from the 3' untranslated regions (UTR) of both genes, and there may be some

species variation in the nucleotide sequences of these UTR. Should this be the case, attempts to reverse transcribe a bovine variant of PG33 or a porcine variant of Ca-CC might have been unsuccessful if the antisense primers were unable to bind these regions of the message.

Message from both genes can, however, be demonstrated in one species. Both Ca-CC and PG33 are expressed in undifferentiated cells from the human colonic carcinoma cell line T84. PG33 is also presumably expressed in non-immortalized human intestinal cells, since it has been isolated from a human infant intestinal cDNA library (see Chapter 3, Cloning Strategies). This suggests, therefore, that the sequence of the 3' untranslated regions may not vary to a large extent between bovine and porcine tissues, if the 3' UTR are not significantly different between these species and human tissues.

Fifty-five cycles of PCR were required to amplify both the Ca-CC and the PG33 message from T84 cells, suggesting that these transcripts are both present in a very low copy number in these cells. While a single product of the expected size appeared when PG33 cDNA was amplified from T84 cells, the amplification of Ca-CC cDNA resulted in multiple products in addition to the expected 766-base pair product Several of these products were smaller than 766 bases, but one was approximately 1600 bases in size, much larger than the expected product. These products had not been observed during the investigation of Ca-CC expression in bovine tissues.

Several possibilities exist which may explain the appearance of these products. The low annealing temperature required may have caused non-specific amplification from the T84 template. Alternatively, the human Ca-CC may occur in one or more isoforms, and these bands may represent splice variants of Ca-CC. These products are also seen after Ca-CC amplification from undifferentiated human airway epithelial (HBE1) cells. These extraneous products may therefore represent a transcript variant particular to human cells, or simply to cells in culture.

Several investigators have also proposed that the differentiation status of a cell may affect the levels of mRNA and protein expression within the cell (Anderson &

Welsh, 1991; Anderson *et al.*, 1992; Morris *et al.*, 1992). In particular, the expression of calcium-regulated (Anderson & Welsh, 1991) and cAMP-regulated (Anderson & Welsh, 1991; Morris *et al.*, 1992) chloride channels in intestinal epithelial cell lines has been shown to depend upon the state of differentiation of the cell. Expression of cAMP-regulated chloride secretion in the intestinal epithelial cell line HT-29 is thought to require polarization of the cell monolayer (Morris *et al.*, 1992), while apical chloride channel activity attributable to calcium agonists is diminished after these cells become polarized (Anderson *et al.*, 1992; Morris *et al.*, 1992). Hence, the reorganization of the plasma membrane as apical and basolateral domains become established during polarization may result in changes in the chloride channel populations of these membrane domains.

If this is the case, differentiation may affect the expression of Ca-CC and PG33 in HBE1 and T84 cells. This hypothesis is supported by the fact that in HBE1 cells, the 838-base pair PG33 target sequence is strongly amplified in differentiated but not in undifferentiated cells. In addition, the extraneous products seen during the amplification of Ca-CC from undifferentiated HBE1 and T84 cDNA are not observed when Ca-CC is amplified from differentiated HBE1 cellular mRNA. The amount of Ca-CC product also appears to be smaller in differentiated cells than in undifferentiated cells. Amplification of Ca-CC from differentiated T84 cells, therefore, might result only in the expected 766-base pair product, and possibly in smaller quantities of amplified product. mRNA from differentiated T84 cells was not available, however, so this hypothesis could not be confirmed.

In summary, the expression of two closely related genes in bovine and in porcine tissues has been compared. Though Ca-CC and PG33 share a similar nucleotide and amino acid sequence, their pattern of tissue expression is different in their respective species of origin: Ca-CC could only be detected in bovine trachea, while PG33 is expressed in porcine trachea, small intestine, and parotid salivary gland. Expression of Ca-CC was not detected in the porcine tissues investigated, nor was PG33 detected in the bovine tissues examined. Ca-CC and PG33 are both expressed in the human cell

lines T84 and HBE1, however, and the level of expression of these genes may depend upon the state of differentiation of these cells.

6.0 General Discussion and Conclusions

This thesis reports the use of an anti-chloride conductance monoclonal antibody to successfully isolate a cDNA from a porcine small intestinal expression library. This original cDNA, while not full-length, was used to isolate a total of 2.3 kb of sequence through the use of conventional oligonucleotide and PCR-based screening of the porcine intestinal library and of a human infant intestinal library. Northern analysis of porcine parotid mRNA indicates that the size of the mature PG33 transcript is approximately 2.7 kb.

In addition to its expression in porcine small intestine, expression of the PG33 gene was also demonstrated in porcine trachea and salivary glands through reverse transcriptase PCR. PG33 mRNA was not detected in several other epithelial tissues, such as pancreas and large intestine, nor was it detected in a number of non-epithelial tissues investigated. In situ hybridization studies confirmed the small intestinal and tracheal expression of the PG33 message. These studies further showed that expression of this mRNA is confined to the mucosal epithelium in the ileum, with particularly high levels of the message observed in certain isolated cells within the villi. In the trachea, PG33 expression was observed in the surface epithelium, and also in high levels in the underlying submucosal glands. The restricted localisation of the PG33 message appears to be consistent with a role in fluid secretion in exocrine epithelial tissues.

The PG33 cDNA sequence is homologous to only two other reported gene sequences: the bovine calcium-regulated chloride channel Ca-CC, and the lung-endothelial adhesion molecule Lu-ECAM-1. The degree of homology between PG33 and Ca-CC prompted a comparative investigation of their gene expression in bovine and porcine tissues. A previous report had indicated that Ca-CC is expressed only in bovine tracheal epithelium (Cunningham *et al.*, 1995), which was confirmed through reverse

transcriptase PCR studies. However, while PG33 is expressed in porcine ileum and parotid gland as well as tracheal epithelia, Ca-CC mRNA could not be detected in bovine ileum or parotid. The expression of these two genes therefore appears to be tissue-specific. Expression of the bovine gene was not, however, detected in porcine tissues, nor the porcine gene in bovine tissues. This may indicate either a species-specific expression of these two genes, or that the experimental techniques were insufficiently sensitive to detect low levels of gene expression.

Expression of both genes was, however, detected in two human cell lines, the colonic carcinoma cell line T84 and the bronchial epithelial line HBE1. Preliminary evidence suggests that expression of the two genes may depend on the relative state of cell differentiation. Because PG33 cDNA was also identified in a human intestinal library, it may be inferred that this gene is expressed in intact human tissues as well as in cultured cells of human origin.

The third member of this family, Lu-ECAM-1, was originally identified as a ligand potentially involved in the adhesion of metastasizing tumor cells to lung vascular endothelium (Zhu *et al.*, 1991; Zhu *et al.*, 1992; Zhu & Pauli, 1993). Lu-ECAM-1 expression has been reported in bovine lung endothelium and in spleen, and this pattern of expression can be distinguished from that of the highly homologous Ca-CC gene (Elble *et al.*, 1997). Chloride channel activity has not yet been reported for Lu-ECAM-1. Since all research efforts to date have focused on its function as a cell adhesion factor, its potential role in ion transport was not apparent, or even considered, until the gene sequence was characterised and the degree of homology to Ca-CC noted.

Given the relationship between the two genes, a number of interesting questions can be raised concerning the physiological role of Lu-ECAM-1. Does this protein in fact exhibit chloride channel activity, and if so, under what circumstances? Furthermore, would such channel activity be associated with the role of Lu-ECAM-1 in cell adhesion and tumor metastasis? Only a few reports to date have linked ion transport and cell adhesion properties within the same protein (Isom *et al.*, 1995; Liu *et al.*, 1996). Speculation as to the purpose of such functional coupling has, therefore, been limited.

The homology of the PG33 cDNA to Ca-CC and Lu-ECAM-1 is less marked but still significant. Regions conserved between the predicted protein products of PG33 and the other genes include the four potential transmembrane domains proposed by Benos' group for Ca-CC (Cunningham *et al.*, 1995). PG33 also contains a number of consensus sites for N-linked glycosylation, similar to those found in Ca-CC and Lu-ECAM-1, and consistent with the findings of the immunoprecipitation studies conducted in this laboratory on intestinal brush-border membrane proteins (Racette *et al.*, 1996). Finally, like Ca-CC, PG33 contains multiple consensus sequences for phosphorylation by serine/threonine protein kinases.

Do these conserved regions indicate conservation of chloride channel function between PG33 and the other two genes? Several lines of evidence would seem to support such a hypothesis. First, the expression of the PG33 gene appears to be restricted to porcine tissues of a classically exocrine nature, and can be specifically localised within these tissues to cells associated with chloride secretion. Second, the conservation of positively charged residues within the potential transmembrane domains of the deduced PG33 protein sequence is consistent with the ionic requirements for a channel that would allow the passage of negatively charged chloride ions. Third, the initial selection criteria for the PG33 cDNA were based on its identification by an antibody which inhibits chloride conductance in intestinal brush-border membrane vesicles.

This final point raises several particularly interesting questions that will need to be addressed in the future. The monoclonal antibody used to select PG33 had been shown to inhibit nearly 100% of conductive ^{36}Cl uptake into HEPES-TMA-equilibrated brush-border vesicles in functional assays (Racette *et al.*, 1996). The population of chloride channels documented in the apical membranes of exocrine epithelial cells is complex, as are the regulatory mechanisms controlling the activity of these channels. That the binding of a single antibody can inhibit the majority of chloride conductive activity is, perhaps, surprising, considering the presence in the brush-border of chloride channels such as CFTR, the ORCC, and ClC-2.

These findings can be reconciled with several different scenarios. If PG33 encodes a chloride channel, it may account for a significant percentage of chloride conductance in the specific intestinal conditions under which conductive uptake was measured, though not necessarily conductance under all possible circumstances. The conductance pathway associated with PG33 has been shown to be activated by cAMP (Forsyth & Gabriel, 1989), which argues that PG33 is physiologically relevant to fluid secretion in exocrine epithelia.

Alternatively, the binding of the antibody to its epitope may physically block chloride conductance, whether through a channel encoded by PG33 or through other conductive pathways in the apical membrane. Many ion channels are known to maintain physical associations with other membrane proteins. An outwardly rectifying chloride channel from bovine tracheal epithelia, for example, copurifies with a regulatory G protein (Ismailov *et al.*, 1996), and CFTR has been shown to interact with both the ORCC and with ENaC (Jovov *et al.*, 1995a; Jovov *et al.*, 1995b; Stutts *et al.*, 1995b; Ismailov *et al.*, 1996). Epitope binding by an antibody may therefore affect not only the activity of the protein to which it binds, but the activity of other proteins associated with that protein.

Future investigations into the function of the PG33 gene product must necessarily address these and other questions. Is PG33 a chloride channel, and if so, what is its physiological role in the tissues in which it is expressed? Completion of the cloning project, and expression of the PG33 cDNA in a mammalian system should be undertaken to resolve this issue. Furthermore, in addition to a potential role as a chloride channel, does PG33, like Lu-ECAM-1, also function in cell adhesion?

If electrophysiological studies do confirm chloride channel activity in PG33, will the single-channel kinetics correlate with any of the known epithelial chloride channels? Although the PG33 DNA sequence eliminates CFTR, Ca-CC, and ClC-2 as potential candidates, a number of other functionally characterised channels are currently under investigation, but have not yet been cloned and sequenced.

Other avenues of investigation include studies of potential regulatory mechanisms

controlling PG33 activity. Ca-CC activity appears to be predominantly stimulated by CaMK II, and D-*myo*-inositol 3,4,5,6-tetrakisphosphate has been shown to inhibit Ca-CC activity (Fuller *et al.*, 1994; Ismailov *et al.*, 1996). Whether PG33 is under similar regulatory control will also need to be determined. As well, in addition to conventional regulation by protein kinases and phosphatases, the notion of coordinate activation of apical and basolateral membrane transport processes by cytoskeletal regulation is gaining prominence, and studies of protein regulation must reflect this.

In conclusion, the process of epithelial chloride secretion, though apparently simple, is in fact the culmination of a highly complex series of events mediated by a large cast of participating proteins under tight control, thereby allowing cells to respond appropriately to changing intracellular and extracellular environments. Knowledge of the individual processes contributing to this response provides not only potential means of therapeutically manipulating these events, but an appreciation of the exquisite redundancy built into the balancing act that maintains the health of a cell.

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IMAGE EVALUATION
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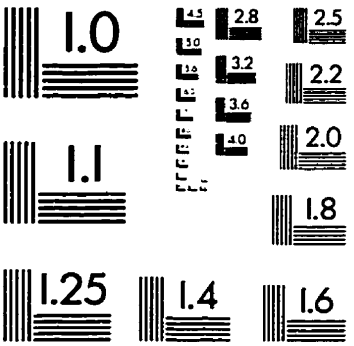

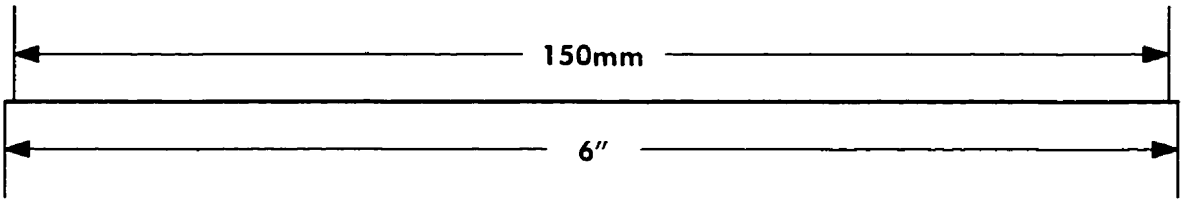

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